Organellar gene expression: Regulation of phage-type RNA polymerase transcript accumulation and analyses of mitochondrial gene copy numbers in *Arabidopsis*

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Let us imagine a palm tree, growing peacefully near a spring, and a lion, hiding in the brush nearby, all of its muscles taut, with bloodthirsty eyes, prepared to jump upon an antelope and to strangle it. The symbiotic theory, and it alone, lays bare the deepest mysteries of this scene, unravels and illuminates the fundamental principle that could bring forth two such utterly different entities as a palm tree and a lion. The palm behaves so peacefully, so passively, because it is a symbiosis, because it contains a plethora of little workers, green slaves (chromatophores) that work for it and nourish it. The lion must nourish itself. Let us imagine each cell of the lion filled with chromatophores, and I have no doubt that it would immediately lie down peacefully next to the palm, feeling full, or needing at most some water with mineral salts.

Mereschkowsky, C. (1905). Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. *Biol. Centralbl.*, **25**: 593–604. English translation in Martin, W., Kowallik, K. V. (1999). Annotated English translation of Mereschkowsky's 1905 paper 'Über Natur und Ursprung der Chromatophoren im Pflanzenreiche'. *Eur. J. Phycol.*, **34**: 287–295.

Abstract

In addition to eubacterial-like multi-subunit RNA polymerases (RNAP) localized in plastids and the nucleus, *Arabidopsis thaliana* contains three phage-like single-unit, nuclear-encoded, organellar RNAPs. The enzymes RpoTp and RpoTm are imported into plastids and mitochondria, respectively, whereas RpoTmp shows dual targeting properties into both organelles. To investigate if expression of the *RpoT* genes is light-dependent, light-induced transcript accumulation of *RpoTm*, *RpoTp* and *RpoTmp* was analyzed using quantitative real-time-PCR in 7-day-old seedlings as well as in 3- and 9-week-old rosette leaves. To address the question whether *RpoT* transcript accumulation is regulated differentially during plant development transcript abundance was measured during leaf development. Additionally, effects of the plants circadian rhythm on *RpoT* transcript accumulation were analyzed. Transcripts of all three *RpoT* genes were found to be strongly light-induced even in senescent leaves and only marginally influenced by the circadian clock. Further analyses employing different photoreceptor mutants and light qualities revealed the involvement of multiple receptors in the light-induction process.

The biogenesis of mitochondria and chloroplasts as well as processes like respiration and photosynthesis require the activity of genes residing in at least two distinct genomes. There have to be ways of intracellular communication between different genomes to control gene activities in response to developmental and metabolic needs of the plant. In this study, it was shown that gene copy numbers drastically increased in photosynthetically inactive *Arabidopsis* seedlings. Mitochondrial DNA contents in cotyledons and leaves ranging in age from 2-day-old cotyledons to 37-day-old senescent rosette leaves were examined. A common increase in senescing rosette leaves and drastic differences between individual genes were found, revealing the importance of an integrative chondriome in higher plant cells.

Keywords: phage-type RNA polymerase**,** light-induction**,** photoreceptors**,** mitochondrial gene copy numbers**,** chondriome

Abstract

Zusätzlich zu der eubakteriellen RNA-Polymerase (RNAP) der Plastiden sind im Zellkern von *Arabidopsis thaliana* drei weitere, phagentypische RNAP kodiert, die jeweils aus nur einer Einheit aufgebaut sind. Die Enzyme RpoTp und RpoTm werden in die Plastiden, bzw. die Mitochondrien transportiert, während RpoTmp in beiden Organellen zu finden ist. Um die Lichtabhängigkeit der *RpoT*-Gene zu untersuchen, wurde die lichtinduzierte Akkumulation ihrer Transkripte in 7-Tage alten Keimlingen, sowie 3- bzw. 9-Wochen alten Rosettenblättern mittels quantitativer *real-time* PCR ermittelt. Die entwicklungsabhängige Regulation der *RpoT*-Transkript-Akkumulation wurde außerdem während der Blattentwicklung analysiert. Zusätzlich wurde der Einfluss des circadianen Rhythmus untersucht. Es stellte sich heraus, dass die Transkriptakkumulation aller drei *RpoT*-Gene stark lichtinduziert war und nur marginalen circadianen Schwankungen unterlag. In weiteren Versuchen mit verschiedenen Lichtrezeptor-Mutanten und unterschiedlichen Lichtqualitäten wurde der Einfluss multipler Rezeptoren auf den Prozess der Lichtinduktion gezeigt.

In den Zellen höherer Pflanzen finden sich drei Genome. Die Biogenese von Chloroplasten und Mitochondrien, sowie lebenswichtige Prozesse, wie Atmung und Photosynthese setzen oftmals die Aktivität von Genen auf mindestens zwei dieser Genome voraus. Eine intrazelluläre Kommunikation zwischen den verschiedenen Genomen ist daher unumgänglich für einen funktionierenden Stoffwechsel der Pflanze. In dieser Arbeit wurde herausgestellt, dass die Zahl mitochondrialer Genkopien in photosynthetisch inaktiven *Arabidopsis*-Keimlingen drastisch erhöht ist. Bei der Untersuchung des DNA-Gehaltes in Proben, die Altersstufen von 2-Tage alten Keimblättern bis hin zu 37-Tage alten, seneszenten Rosettenblättern umfassten, fand sich ein deutlicher Anstieg der Kopienzahlen in älteren Rosettenblättern. Außerdem unterschieden sich die Kopienzahlen der untersuchten Gene zum Teil erheblich voneinander.

Schlagworte: Phagentyp-RNA-Polymerasen**,** Lichtinduktion**,** Photorezeptoren**,** mitochondriale Genkopienzahlen**,** Chondriom

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Zusammenfassung

Der organelläre Transkriptionsapparat höherer Pflanzen ist äußerst komplex. Zusätzlich zu der aus mehreren Untereinheiten aufgebauten, eubakteriellen RNA-Polymerase (RNAP), die in den Plastiden zu finden ist, gibt es in dikotyledonen Pflanzen, wie *Arabidopsis thaliana*, drei weitere, kernkodierte RNAPs. Diese RNAPs bestehen aus nur einer Einheit und sind von entsprechenden Enzymen der Bakteriophagen abgeleitet. Die Gene, die für die Polymerasen kodieren gehören zur *RpoT*-Genfamilie, welche in beinahe allen Eukaryoten zu finden ist. Während RpoTp und RpoTm in die Plastiden, bzw. die Mitochondrien transportiert werden, ist ein drittes Enzym, RpoTmp, in beiden Organellen zu finden. Über die Regulation von Expression und Transkription der *RpoT*-Gene ist bislang nicht viel bekannt. Um eine lichtabhängige Expression zu untersuchen, wurde in dieser Arbeit mittels quantitativer *real-time* PCR die lichtinduzierte Transkriptakkumulation von *RpoTm*, *RpoTp* und *RpoTmp* in 7-Tage alten Keimlingen, sowie 3- und 9-Wochen alten Rosettenblättern untersucht. Um der Frage nach einer entwicklungsabhängigen Regulation der *RpoT*-Transkriptakkumulation nachzugehen, wurde diese auch während der Blattentwicklung gemessen. Weiterhin wurde der Einfluss des circadianen Rhythmus auf die Akkumulation der *RpoT*-Transkripte analysiert. Anhand der gewonnen Daten konnte eine stark lichtinduzierte Akkumulation der Transkripte alles drei Polymerasen nachgewiesen werden. Der Einfluss der circadianen Rhythmik dagegen war nur marginal. In weiteren Versuchen wurde mithilfe verschiedener Lichtrezeptor-Mutanten und unterschiedlicher Lichtqualitäten versucht, einen genaueren Einblick in die Vorgänge zu erhalten, die bei der lichtinduzierten Akkumulation der *RpoT*-Transkripte eine Rolle spielen. Die Ergebnisse machten deutlich, dass für die Lichtinduktion ein Netzwerk verschiedener Rezeptoren benötigt wird.

Aufgrund ihrer evolutionären Abstammung von einst frei lebenden Prokaryoten besitzen Plastiden und Mitochondrien immer noch ihre eigene DNA. Daher existieren in Pflanzenzellen drei verschiedene Genome. Die Biogenese der Organellen, sowie viele, oftmals lebenswichtige Prozesse, wie Zellatmung und Photosynthese, setzen die Aktivität von Genen auf mindestens zwei dieser Genome voraus. Eine ausgeprägte intrazelluläre Kommunikation ist daher für die Entwicklung und einen funktionierenden Stoffwechsel der Pflanze unumgänglich. Um einen Einblick in die Komplexität dieser Vorgänge zu gewinnen, wurden in dieser Arbeit spectinomycin-behandelte, weiße *Arabidopsis*-Keimlinge ohne funktionelle Chloroplasten bezüglich der mitochondrialen Genexpression analysiert. Quantitative real-time PCR-Analysen zeigten deutlich erhöhte mitochondriale Transkriptmengen, sowie eine größere Zahl mitochondrialer Genkopien in den photosynthetisch inaktiven Pflanzen. Über den Einfluss mitochondrialer Genkopienzahlen auf die mitochondriale Genexpression ist bislang nur wenig bekannt. Um dieses Phänomen weiter zu untersuchen, wurden in dieser Arbeit Proben, die Altersstufen von 2-Tage alten Keimblättern bis hin zu 37-Tage alten, seneszenten Rosettenblättern umfassten, auf ihren mitochondrialen DNA-Gehalt hin untersucht. Die Zahl der Kopien aller untersuchten Gene lag deutlich unter der geschätzten Zahl der Mitochondrien pro Zelle. Ein Anstieg der Kopienzahlen während der frühen Seneszenz älterer Rosettenblätter konnte für alle untersuchten Gene beobachtet werden. Außerdem unterschieden sich die Kopienzahlen einzelner Gene zu Teil erheblich voneinander. Diese Daten deuten auf das Vorhandensein von subgenomischen Molekülen und deren differentielle Amplifikation hin und machen die Bedeutung eines integrativen Chondrioms in Zellen höherer Pflanzen deutlich. Der in den weißen Pflanzen beobachtete Anstieg der mitochondrialen Genkopienzahlen und Transkriptmengen ist nur mithilfe einer komplexen Vernetzung der Signalwege von Plastiden, Mitochondrien und Zellkern möglich.

Summary

The transcription machinery of higher plant organelles is very complex. In addition to eubacterial-like multi-subunit RNA polymerases (RNAP) localized in plastids and the nucleus, dicotyledonous plants, like *Arabidopsis thaliana,* contain three phage-like, single-unit, nuclear-encoded, organellar RNAPs. The genes coding for these enzymes belong to the *RpoT* gene family, which is found throughout the eukaryotic kingdom. RpoTp and RpoTm are imported into plastids and mitochondria, respectively, whereas a third polymerase, RpoTmp, shows dual targeting properties into both organelles. To date, not much is known about the regulation of transcription and expression of the *RpoT* genes. To investigate if their expression is light-dependent, light-induced transcript accumulation of *RpoTm*, *RpoTp* and *RpoTmp* was analyzed in 7-day-old seedlings as well as in 3- and 9-week-old rosette leaves using quantitative real-time-PCR. To address the question whether *RpoT* transcript accumulation is furthermore regulated differentially during plant development transcript abundance was measured during leaf development. Additionally, effects of the plants circadian rhythm on *RpoT* transcript accumulation were analyzed. The study revealed transcript accumulation of all three *RpoT* genes to be strongly light-induced in young seedlings and even in senescent leaves. However, transcript accumulation was only marginally influenced by the circadian clock. To get an insight into the pathways that are responsible for the light-induced accumulation of *RpoT* transcripts, further analyses employing different photoreceptor mutants and light qualities were carried out. The obtained data revealed participation of a network of multiple photoreceptors and downstream pathways in the light-induction process.

Due to the evolutionary origin of plastids and mitochondria from once free-living prokaryotes, these organelles still contain their own DNA. Plant cells thus contain three genomes. The biogenesis of mitochondria and chloroplasts as well as many vital processes including respiration and photosynthesis require the activity of genes residing in at least two of these genomes. There have to be ways of intracellular communication between different genomes to control gene activities in response to developmental and metabolic needs of the plant. To address this issue, spectinomycin-treated, white *Arabidopsis* seedlings lacking functional chloroplasts were analyzed regarding mitochondrial gene expression. Quantitative real-time PCR analyses revealed higher mitochondrial transcript accumulation as well as broadly increased numbers of mitochondrial gene copies in photosynthetically inactive plants. As yet, little is known about the impact of mitochondrial gene copy numbers on the expression of mitochondrial genes. To further investigate this issue, in this study, the mitochondrial DNA content in cotyledons and leaves ranging in age from 2-day-old cotyledons to 37-day-old senescent rosette leaves was examined. Overall copy numbers of the analyzed genes were notably below the predicted number of mitochondria per cell. A common increase in gene copy numbers was obvious in older rosette leaves showing first signs of senescence. Furthermore, drastic differences between individual genes were found. The data thus suggest differential amplification of subgenomic molecules and reveal the importance of an integrative chondriome in higher plant cells. The observed effects during development and in white seedlings require the existence of a signaling network between mitochondria, plastids and the nucleus in which changes in the energy demand of the plant are sensed and accordingly taken care of.

1 Introduction

The transition from prokaryotes to eukaryotes was one of the most profound changes in the evolutionary history of life. However, the exact scenario for the emergence of the first eukaryotic cell is still unsettled and hotly debated in current literature. Multiple competing hypotheses presenting broadly different concepts for the origin of eukaryotes have arisen recently (Dagan und Martin, 2007; Embley und Martin, 2006; Kurland *et al.*, 2006; Martin und Koonin, 2006; Martin und Muller, 1998; Poole und Penny, 2007). To date it has not been possible to ultimately proof one or the other theory. Thus, further research in the field of molecular evolution is needed to address this fundamental question. According to the hydrogen hypothesis formulated by Martin and Müller (1998), eukaryotes arose through a single endosymbiotic event, in which the host was an autotrophic, hydrogen-dependent archaebacterium, while the eubacterial symbiont produced molecular hydrogen as a waste product of its anaerobic, heterotrophic metabolism (Martin und Muller, 1998). There would thus be two primary lineages of life, archaebacteria and eubacteria, while eukaryotes were a chimeric lineage originating from the symbiosis of two prokaryotes (Esser und Martin, 2007; Martin und Muller, 1998; Pisani *et al.*, 2007).

1.1 The origin of organelles and their roles in higher plants

1.1.1 Mitochondria

The acquisition of mitochondria came along with the origin of the eukaryotic lineage. In a largely accepted scenario, mitochondria evolved from progenitors of today's α-proteobacteria in a single endosymbiotic event over 1.5 billion years ago (Gray *et al.*, 1999; Margulis, 1970; Martin *et al.*, 2001; Martin und Muller, 1998).

Mitochondria were discovered in 1886 by the German pathologist Richard Altmann. He observed similarities in size, shape and staining properties between these "cell granules" and free-living bacteria and already suggested that mitochondria might derive from prokaryotic ancestors (Altmann, 1890). However, it took another thirty-seven years until Ivan Wallin postulated an endosymbiotic origin of mitochondria (Wallin, 1927). Later, Lynn Margulis formalized the theory of endosymbiosis, demonstrating that plastids and mitochondria derive from bacterial endosymbionts (Margulis, 1970; Margulis, 1971).

Mitochondrial morphology may vary to a great extent in different organisms and tissues depending on cell type and physiological state. However, typically, the organelles are 1 – 2

µm long and 0.1 – 0.5 µm in diameter (Logan, 2006). While mitochondria in mammals and yeast are often tubular and form reticular networks (Karbowski und Youle, 2003; Stevens, 1977) higher plant mitochondria usually are discrete, spherical organelles (Logan und Leaver, 2000; Logan, 2006). They display high motility and undergo frequent fusion and fission (Arimura *et al.*, 2004; Logan und Leaver, 2000; Logan, 2003). Thereby, the chondriome of higher plant cells builds a network that can be termed a discontinuous whole (Logan, 2006); see 1.3).

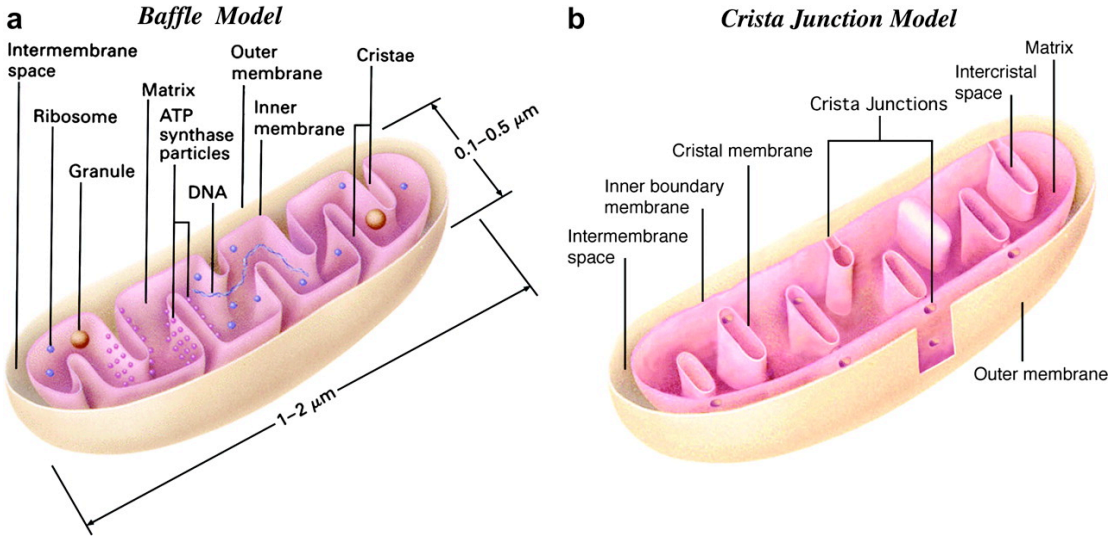


Figure 1: Model of a mitochondrion with typical membrane structures.
The "baffle" model (a) was developed by Palade in the 1950s and was broadly accepted until recently. Modern 3D visualization techniques such as electron tomography have led to the new "crista junction model" (b). It supplants the baffle model in all animal mitochondria examined so far. According to the latter model the intercristal space is connected to the inner membrane by narrow tubular openings called crista junctions rather than by the wide ports as implicated by the baffle model. Taken from Logan, 2006.

Mitochondria are surrounded by a double membrane which contains two complex protein import apparatuses, named TIM and TOM, for translocase of the inner and the outer mitochondrial membrane, respectively (Heins *et al.*, 1998; Jansch *et al.*, 1998; Murcha *et al.*, 2003; Pfanner *et al.*, 2004; Truscott *et al.*, 2001; Whelan und Glaser, 1997). The inner mitochondrial membrane which encloses the matrix space is folded into cristae (see Fig. 1; (Mannella, 2006; Palade, 1957). Components of the mitochondrial electron transport chain are an integral part of the inner membrane, as are other enzymes such as ATP synthase and succinate dehydrogenase (Bowsher und Tobin, 2001).

Altmann already suggested in 1886 that mitochondria were involved with cellular oxidation (Hughes, 1959). In 1949, Kennedy and Lehninger were finally able to show that

the primary role of mitochondria is the allocation of oxidative energy to the cell (Kennedy und Lehninger, 1949).

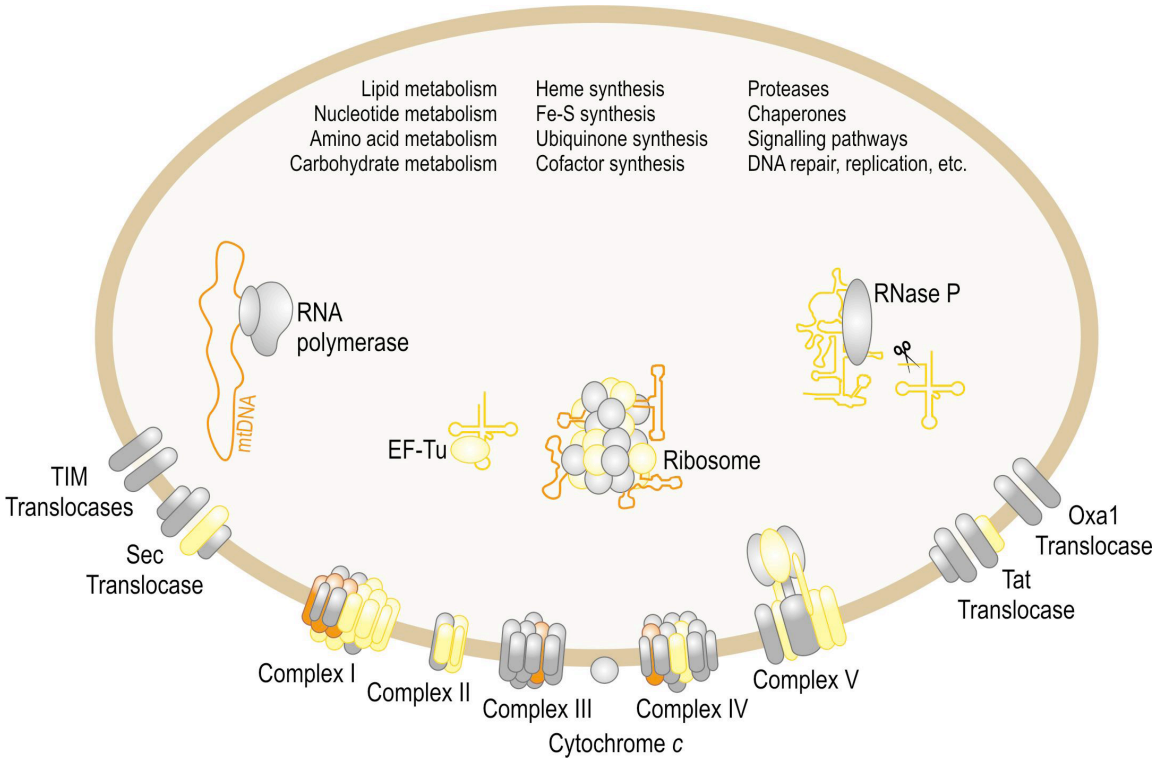


Figure 2: Biological processes in mitochondria.
The majority of mitochondrial functions are shown; only the mitochondrial matrix and inner membrane are depicted. Most mitochondrial components are nucleus-encoded (examples shown in grey), and the majority of mitochondrial processes have exclusively nucleus-encoded constituents (listed). Yellow symbols correspond to proteins and RNAs encoded by the mtDNA in some eukaryotes but by the nuclear genome in others. Very few components are specified by the mtDNA in all organisms (orange). Displayed components are involved in electron transport and oxidative phosphorylation (complexes I-V and cytochrome c), protein import and insertion into the inner membrane (TIM), protein export from the matrix and insertion into the inner membrane (Tat, Sec, Oxa1), mtDNA transcription (RNA polymerase), tRNA 5'-end processing (RNase P), protein synthesis (ribosomes and elongation factor EF-Tu). Modified from (Burger *et al.*, 2003).

Besides this well established function, the synthesis of ATP through an electron transport chain (Saraste, 1999), higher plant mitochondria were shown to be responsible for a wide range of additional metabolic processes (see Fig. 2). The organelles are known to play critical roles in the synthesis of vitamin co-factors, such as ascorbate (Millar *et al.*, 2003), folate (Mouillon *et al.*, 2002; Ravanel *et al.*, 2001), biotin (Picciocchi *et al.*, 2003) and lipoic acid (Yasuno und Wada, 2002), as well as in the synthesis and degradation of fatty acids (Baker *et al.*, 2006; Focke *et al.*, 2003; Gueguen *et al.*, 2000) and the lipoylation of proteins (Ewald *et al.*, 2007). Another very important process, the synthesis of Fe-S clusters, partly takes place in mitochondria (Kushnir *et al.*, 2001). Additionally, a considerable part

of the amino acid metabolism is associated with mitochondria (Nunes-Nesi und Fernie, 2007). Furthermore, higher plant mitochondria are involved in photorespiration (Douce und Neuburger, 1999), cell signaling (Bianchi *et al.*, 2005; Logan und Knight, 2003) and programmed cell death (Jones, 2000; Vanlerberghe *et al.*, 2002; Youle und Karbowski, 2005).

1.1.2 Plastids

The first trusted description of chloroplasts, namely those of the green alga *Spirogyra* comes from Thonis (Anthony) van Leeuwenhoek and dates back almost exactly 335 years. He coincidentally found them in water samples from the Dutch *Berkelse Mere* with his self-made single-lens microscope and mentioned his observations in a letter to the Royal Society of London (Dobell, 1932).

In 1883, German botanist Andreas Franz Wilhelm Schimper was the first scientist to postulate a prokaryotic origin of chloroplasts (Schimper, 1883). A few years later Mereschkowsky developed the theory of endosymbiosis in which he proposed that the *chromatophores* (chloroplasts) were formerly free-living organisms which entered the cytoplasm of the cell to become symbiotic organelles (Martin und Kowallik, 1999; Mereschkowsky, 1905). As in the case of mitochondria, the endosymbiotic theory was formalized in the 1970s by Lynn Margulis (Margulis, 1970; Margulis, 1971).

It is now commonly accepted that plastids arose through symbiosis of a eukaryotic host (that already possessed mitochondria) and a photosynthetic prokaryote ancestral to present-day cyanobacteria (Douglas und Turner, 1991; Hess *et al.*, 2001; Nelissen *et al.*, 1995; Palmer, 2003; Turner *et al.*, 1999). This presumably monophyletic event was designated primary endosymbiosis (Gray, 1992; McFadden, 2001). Recent analyses suggest that the primary endosymbiosis was established approximately 1.5 billion years ago (Hedges *et al.*, 2004; Martin *et al.*, 2003; Yoon *et al.*, 2004). In the evolution of plants and algae more complex events occurred, leading to secondary and tertiary plastids via the engulfment of unicellular eukaryotic photoautotrophs by eukaryotic cells (see Fig. 3; (Delwiche, 1999; Hjorth, 2004; McFadden, 2001; McFadden *et al.*, 1994).

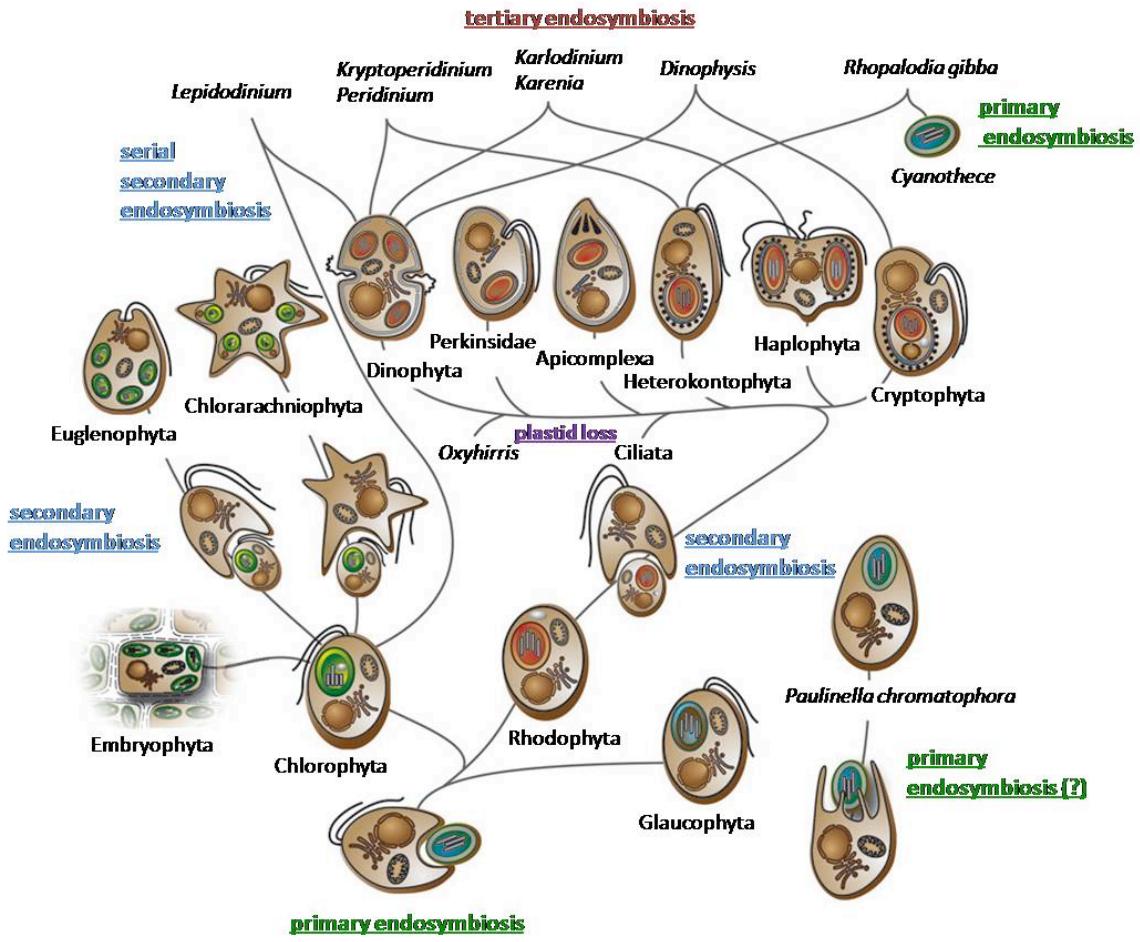


Figure 3: Schematic representation of plastid evolution.
The initial eukaryotic endosymbiosis led to formation of three lineages with primary plastids: glaucophytes, rhodophytes and chlorophytes, the latter giving rise to all land plants. Uptake of green and red algae led to secondary endosymbiosis in the case of euglenophytes, chlorarachniophytes and the very diverse but most probably monophyletic chromalveolates. Different dinophytes have replaced their secondary plastids with a green alga either by serial secondary or even tertiary endosymbiosis. The heterokontophyte *Rhopalodia gibba* engulfed a cyanobacterial *Cyanothece* species and reduced it to so-called speroid bodies which are used for nitrogen fixation. Some species (Ciliates, *Oxyhirris*) lost their plastids. Whether the case of *Paulinella chromatophora* represents a true primary endosymbiosis is still debated. Modified from Gould *et al.*, 2008.

Plastids are present, with very few exceptions, in all algae, mosses, ferns, gymno- and angiosperms (Gould *et al.*, 2008) and additionally in some unicellular parasites (Obornik *et al.*, 2008; Waller und McFadden, 2005) and marine mollusks (Hoffmeister und Martin, 2003; Rumpho *et al.*, 2001; Rumpho *et al.*, 2008). They are moreover a diverse group of organelles and are represented by over a dozen different variants that are derived either by development or by evolution (Gould *et al.*, 2008; Kirk und Tilney-Bassett, 1978; Reyes-Prieto *et al.*, 2007; Thomson und Whatley, 1980).

Chloroplasts, as all other forms of plastids developmentally originate from a small vesicle called proplastid. They are usually lens-shaped, between 5 and 10 µm in diameter and 3 - 4 µm in thickness (López-Juez und Pyke, 2005; Staehelin, 2003). The surrounding double membrane envelope contains transport systems to assure passage of metabolites and proteins. Transport via the outer membrane pores is less discriminatory than translocation over the inner membrane, but both contain highly specific import apparatuses. These complexes are called Toc and Tic for translocase of the outer and inner plastid membrane, respectively (Hörmann *et al.*, 2007; Jarvis und Soll, 2002; Kalanon und McFadden, 2008; Soll und Schleiff, 2004).

Chloroplasts are well-known for being the sites of photosynthesis (Allen, 2005), but they carry out multiple additional tasks and are central hubs in plant metabolism (Neuhaus, 2000). They play major roles in a lot of different important and often essential processes including the synthesis of fatty acids (Bao *et al.*, 2000; Beisson *et al.*, 2003; Branen *et al.*, 2003), aromatic and non-aromatic amino acids (Hell *et al.*, 2002; Herrmann und Weaver, 1999; Ireland und Lea, 1999; Ward und Ohta, 1999), isoprenoids (Laule *et al.*, 2003; Lichtenthaler *et al.*, 1997), tetrapyrroles (Porra *et al.*, 1983; Vavilin und Vermaas, 2002) Fe-S clusters (Balk und Lobreaux, 2005; Takahashi und Tokumoto, 2002) starch (Kossmann und Lloyd, 2000; Niittylä *et al.*, 2004; Zeeman *et al.*, 2002) and sucrose (Huber und Huber, 1996; Koch, 2004).

Due to these crucial roles in many metabolic pathways, also non-photosynthetic cells are dependent on plastids. Plants can harbor a lot of morphologically and functionally distinct types of plastids. They all originate from the small colorless *proplastids* which are found in meristematic tissues and embryos. Proplastids are only 0.2 – 1 µm in diameter and contain very limited internal membrane vesicles (Pyke und Leech, 1992; Waters und Pyke, 2004). So called *amoeboid plastids* are larger than proplastids with more developed internal membranes and show a highly variable morphology. They are often found in root cells and very young leaf cells. The main function of a very common type of plastids, the *amyloplasts*, is the storage of starch. Amyloplasts also possess an active oxidative pentose phosphate pathway that generates energy to assimilate nitrogen (Neuhaus, 2000). These plastids are often major constituents of root cells and storage organs like tubers, cotyledons and seed endosperm (Waters und Pyke, 2004). In addition to starch, plastids can also store other metabolites, such as lipids. In oil-accumulating storage organs, such as oilseeds, they are called *elaioplasts*. Those storing aromatic oils and often found in secretory hairs are referred to as *leucoplasts* (López-Juez und Pyke, 2005). The colorful appearance of flower

petals or fruits in many plant species is caused mainly by carotenoids and xanthophylls, which are also stored in specialized plastids, called *chromoplasts* (Bramley, 2002; Waters und Pyke, 2004; Weston und Pyke, 1999). In leaf cells, normally containing chloroplasts, light is needed for the conversion of protochlorophyllide into chlorophyll. Without sufficient amount of light these cells develop a special form of chlorophyll-less plastids that were called *etioplasts*, as dark-grown seedlings are termed etiolated. Upon illumination, etioplasts will eventually develop thylakoids with photosynthetic complexes and become green, photosynthetically active chloroplasts (López-Juez und Pyke, 2005). All the different plastid types can be converted into each other under certain conditions (see Fig. 4). An exception is the *gerontoplast*, which is an irreversible degradation product of senescing chloroplasts (Biswal *et al.*, 2003).

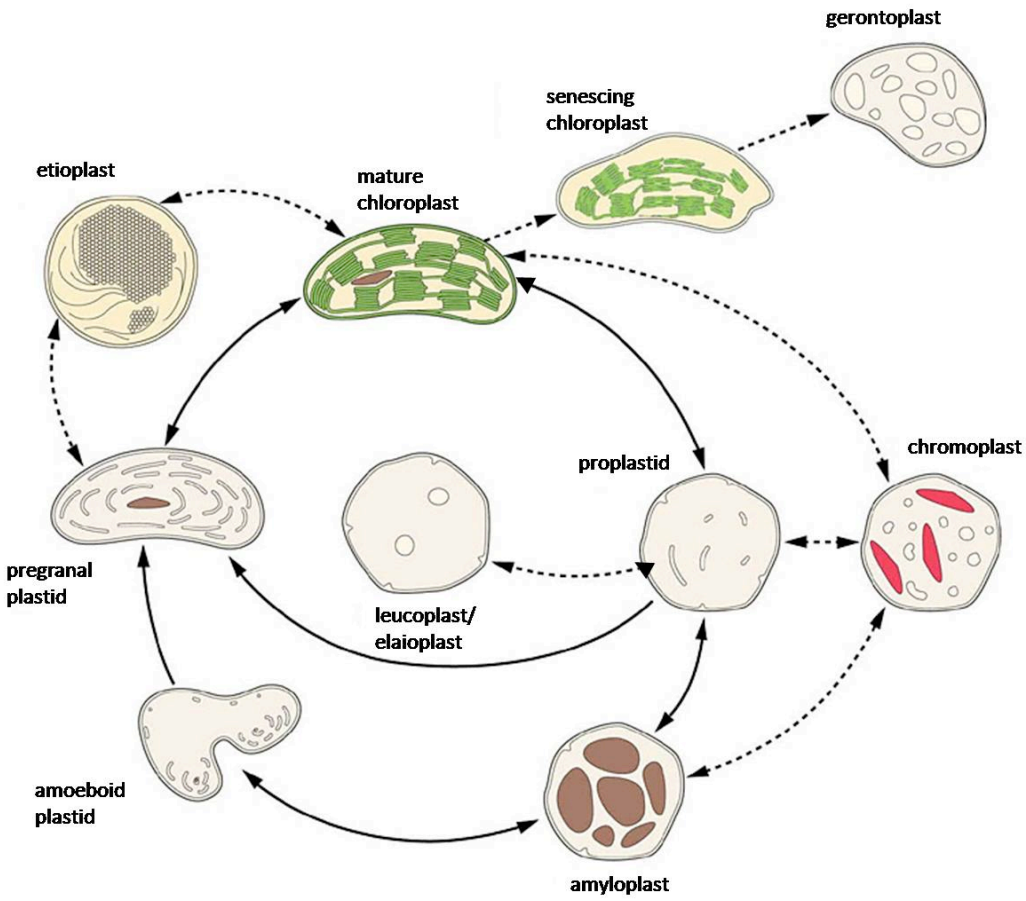


Figure 4: Schematic outline of major types of plastids.
Different types of plastids that, under certain conditions and in certain cell types, are derived directly or indirectly from proplastids and their relations are shown. Modified from Buchanan *et al.*, 2000

1.2 Higher plant organellar genomes

1.2.1 The chondrome of higher plants

As mitochondria are descendants of endosymbiotic, α -proteobacteria-like progenitors (Gray *et al.*, 1999), they still possess a genome which is called the chondrome. However, mitochondria of contemporary organisms contain considerably fewer genes than the ancestral bacteria. Instead, thousands of mitochondrial proteins are encoded in the nucleus (Heazlewood *et al.*, 2004; Millar *et al.*, 2005).

This is explained by the fact that there has been massive gene transfer from mitochondria to the nucleus of the host cell during evolution (Adams und Palmer, 2003; Henze und Martin, 2001; Martin, 2003; Martin und Herrmann, 1998). In most animals this led to the retention of a fairly stable number of about 13 protein-coding genes, together with various components of translation. Gene content in mitochondrial genomes of higher plants is more variable and usually comprises between 54 and 57 known genes (Clifton *et al.*, 2004; Kubo *et al.*, 2000; Notsu *et al.*, 2002; Sugiyama *et al.*, 2005; Unseld *et al.*, 1997). Higher plant chondromes exhibit differential gene losses, indicating that the process of gene transfer to the nucleus continues to present day (Adams und Palmer, 2003; Timmis *et al.*, 2004).

Whereas mitochondrial genomes of animals range in size from around 15 to 18 kbp and those of yeast from 18 to more than 100 kbp, plant chondromes are substantially lager and range from 208 kbp in white mustard (*Brassica hirta*) to over 2400 kbp in muskmelon (*Cucumis melo*; (Kubo und Newton, 2008; Palmer und Herbon, 1987; Ward *et al.*, 1981). However, despite plant mitochondria containing considerably bigger genomes, they do not encode a proportionately higher number of genes (Bullerwell und Gray, 2004; Burger *et al.*, 2003). For example, the mitochondrial genome of *Arabidopsis thaliana* (~ 367 kbp; Fig. 3) is 22 times as large as the human mitochondrial genome (16.6 kbp) but encodes only 2.5 times as many genes (33 polypeptides in *A. thaliana*, 13 in humans; (Anderson *et al.*, 1981; Unseld *et al.*, 1997). The large sizes of plant chondromes are attributed to frequent duplications and large intergenic regions resulting from incorporation of non-coding sequence, introns and unidentified open reading frames (ORFs) from plastids and the nucleus and through horizontal gene transfer (Clifton *et al.*, 2004; Kubo und Newton, 2008; Marienfeld *et al.*, 1999; Palmer *et al.*, 2000; Unseld *et al.*, 1997).

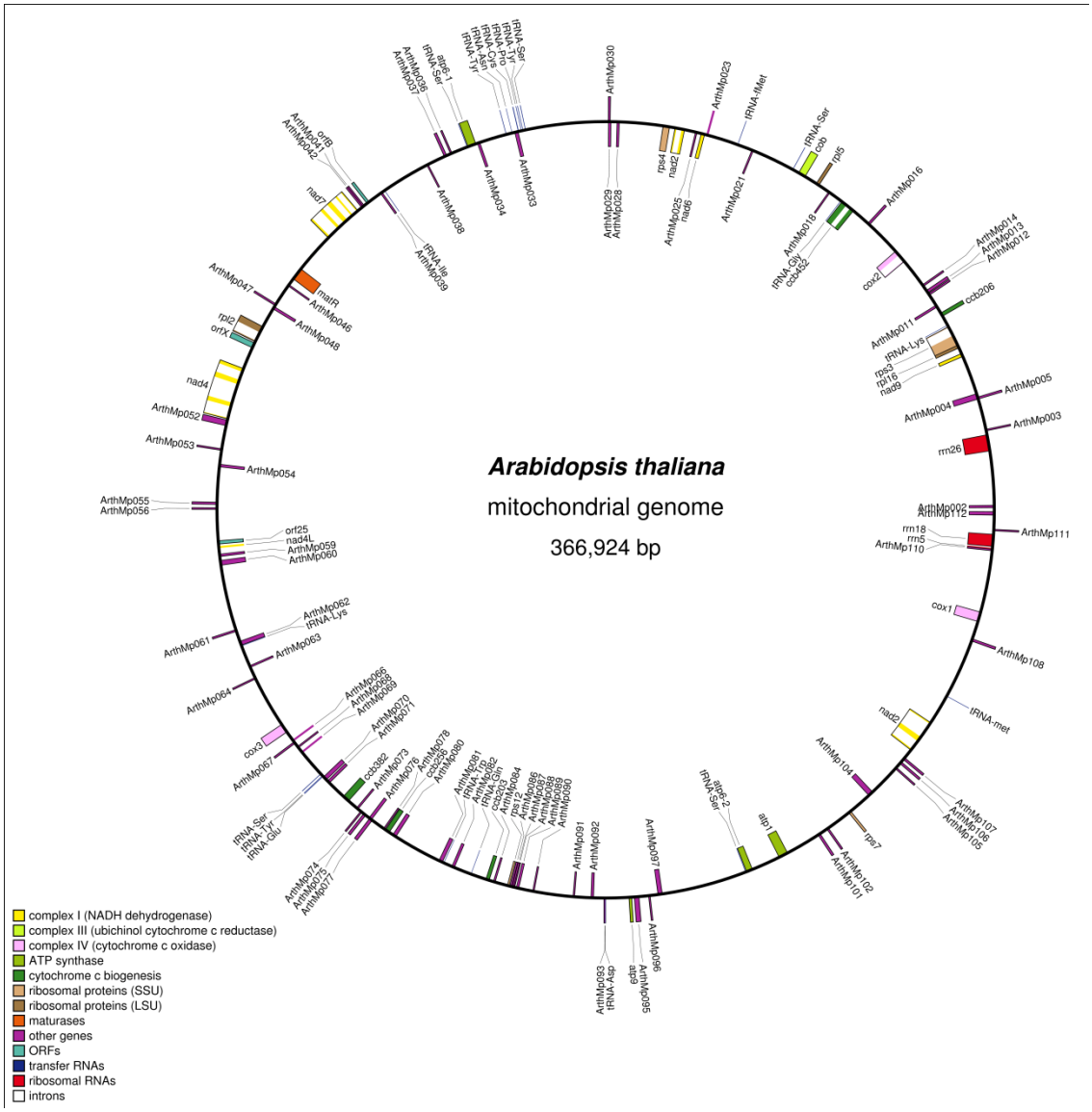


Figure 5: Map of the mitochondrial genome of *Arabidopsis thaliana*.
Identified genes encoding polypeptides, tRNAs and rRNAs, as well as introns, are indicated. Made with OGDRAW v1.1 (Lohse *et al.*, 2007).

The genes that are located on the mitochondrial genome of higher plants differ slightly between species, but generally encode products that are directly or indirectly involved in oxidative phosphorylation and ATP production (Clifton *et al.*, 2004; Handa, 2003; Kubo *et al.*, 2000; Notsu *et al.*, 2002; Ogihara *et al.*, 2005; Sugiyama *et al.*, 2005; Unseld *et al.*, 1997). They may be organized in gene clusters or dispersed over the complete genome, giving rise to both mono- and polycistronic transcripts. Maturation of these transcripts involves multiple processing steps, such as RNA editing (Bentolila *et al.*, 2008; Handa, 2003; Mower und Palmer, 2006; Mulligan *et al.*, 2007; Takenaka *et al.*, 2008), generation of secondary 5' and 3' ends (Forner *et al.*, 2007) and removal of group II introns (Bonen,

2008). Mitochondrial DNA is usually organized in membrane-associated nucleoids, which are located in the matrix (Dai *et al.*, 2005; Fey *et al.*, 1999; Sasaki *et al.*, 1998). The proteins associated with these chromatin-like structures in plants are still to be identified, however (Sakai *et al.*, 2004).

Fully sequenced plant mitochondrial genomes are commonly assembled as a circular chromosome (see Fig. 5), which in earlier studies was termed the "master circle" (Lonsdale *et al.*, 1988), although recent studies have shown a different structure *in vivo*. Within most plant tissues the mtDNA seems to exist as a heterogeneous population of mostly linear, often branched molecules. Smaller circular molecules are also thought to exist, albeit to a much lesser extent (Backert und Börner, 2000; Bendich, 1996; Oldenburg und Bendich, 1996). This complex structure of the chondrome arises by frequent and active homologous recombination at large repeat regions, creating a multipartite, highly redundant organization of subgenomic molecules (Fauron *et al.*, 1995). A second type of recombination characteristic for plant mitochondria involves sporadic, low frequency illegitimate events at smaller repeats, resulting in substoichiometric DNA molecules that may replicate autonomously and eventually lead to cytoplasmic male sterility (Abdelnoor *et al.*, 2003; Andre *et al.*, 1992; Lonsdale *et al.*, 1988; Small *et al.*, 1987; Small *et al.*, 1989). Recombination activity and maintenance of subgenomic molecules is supposed to be under nuclear control (Abdelnoor *et al.*, 2003; Shedge *et al.*, 2007).

1.2.2 The plastome

Plastids emerged from the association of a mitochondrion-containing eukaryote and an ancestor of today's cyanobacteria in a single endosymbiotic event (Deusch *et al.*, 2008; Martin *et al.*, 2002), followed by diversification into the different primary, secondary and tertiary plastids we know today (see Fig. 3; (Martin *et al.*, 2002). Therefore, plastid genomes are highly conserved between species.

As in the case of mitochondria, most of the genes originally encoded by the free-living progenitor of plastids were transferred to the nucleus or lost in the course of evolution (Adams und Palmer, 2003; Henze und Martin, 2001; Martin, 2003; Martin und Herrmann, 1998). Plastid genomes of higher plants assemble into a circular chromosome ranging between 120 and 160 kbp in size, the only exception being some non-photosynthetic parasites, like *Epiphagus virginiana*, whose plastids contain only around 70 kbp (Bungard, 2004; Krause, 2008; Wakasugi *et al.*, 2001). The number of genes encoded on plastomes

ranges from 120 to 135 (see the Organelle Genome Megasequencing Program, http://megasun.bch.umontreal.ca/ogmp, for a complete set of available genomes), of which approximately 80 code for proteins (López-Juez und Pyke, 2005; Martin *et al.*, 2002). The chloroplast genome encodes several polypeptides important for photosynthesis, including NADH dehydrogenase, Rubisco, ATP synthase, cytochrome, photosystem I and photosystem II components (De Las Rivas *et al.*, 2002; López-Juez und Pyke, 2005; Sugiura *et al.*, 1998). Additionally, subunits of the eubacterial-type RNA polymerase, a few proteins of other functions as well as ribosomal and transfer RNAs are encoded on the plastome. Many genes are organized in operons and expressed as polycistronic units requiring post-transcriptional modifications such as trans-splicing and RNA editing (Maier *et al.*, 2008; Sugiura, 1992; Tillich *et al.*, 2006).

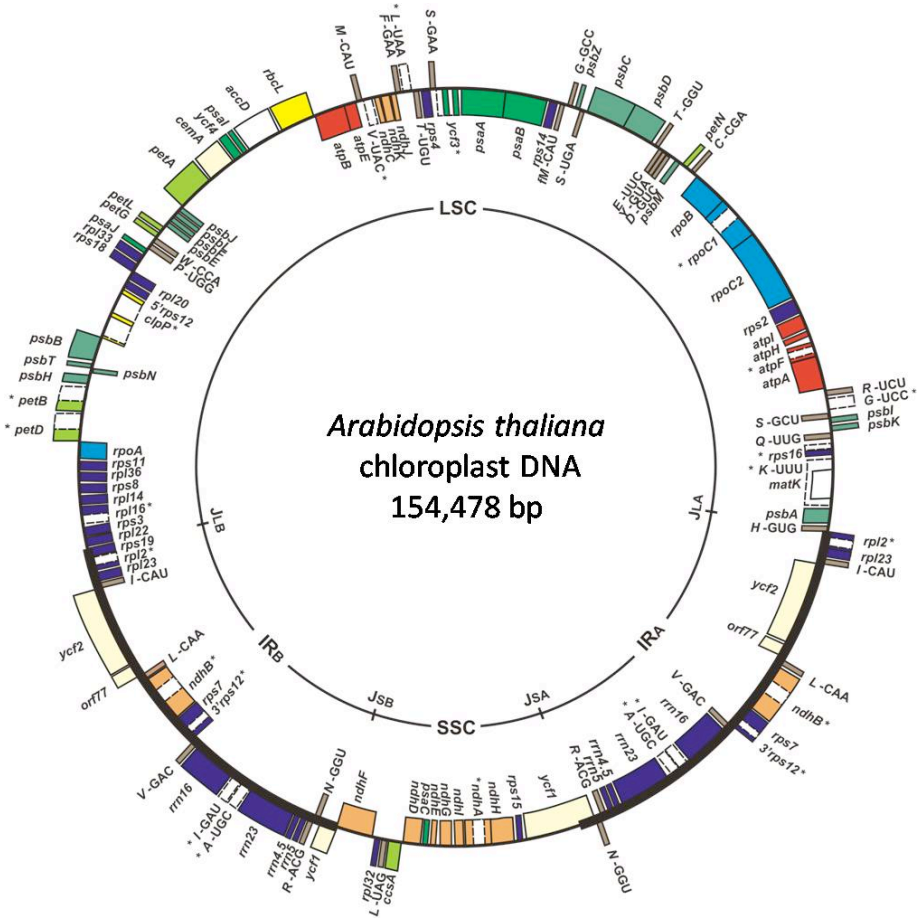


Figure 6: Map of the plastid genome of *Arabidopsis thaliana*.
The two inverted repeat regions (IR_A and IR_B) of 26,264 bp are separated by the large (LSC, 84,170 bp) and small (SSC, 17,780 bp) single-copy regions. The *Arabidopsis* plastome comprises ~ 120 genes encoding 87 proteins, 4rRNAs and 30 tRNAs (37 tRNA genes). Genes and ORFs are color-coded according to their biological function. Genes outside the ring are transcribed from right to left, those inside from left to right. Genes containing introns are marked with an asterisk; introns are depicted by dashed boxes. From Sato *et al.*, 1999.

A characteristic of the plastid genome is its structural organization as a large and a small single-copy region linked by two identical, inverted copies of a large DNA section, called inverted repeats A and B (Kolodner und Tewari, 1979; Wang *et al.*, 2008).

Plastids are highly polyploid. While proplastids contain around 20 genome copies, resulting in approximately 400 copies per meristematic cell (Sugiura, 1992), plastome copy number in rosette leaf cells lies between 1000 and 1700 without significant variation during development and senescence (Zoschke *et al.*, 2007).

Similar to mitochondrial DNA (see 1.2.1), plastid DNA is organized in chromatin-like structures called nucleoids, which are associated with the inner envelope membrane through specific protein complexes (Sato *et al.*, 2003; Terasawa und Sato, 2009).

1.3 The plant chondriome

The collectivity of mitochondria inside one cell is called the chondriome. In yeast cells and most animal cell types, the chondriome is frequently organized into networks that build long tubules or reticula (Bereiter-Hahn und Vöth, 1994; Karbowski und Youle, 2003; Mozdy *et al.*, 2000). In higher plants, the structure of the chondriome contrasts with that in most other eukaryotes, consisting mainly of physically discrete, often sausage-shaped organelles (Logan, 2006; Lonsdale *et al.*, 1988). It has therefore been suggested to term the plant mitochondrial chondriome a “discontinuous whole” as opposed to the “dynamic syncytium” often found in other eukaryotes (Logan, 2006).

The mitochondrial genome is relatively large and complex in its structure (Kubo und Newton, 2008) and contains multiple repeat regions that are highly active hotspots of recombination (Fauron *et al.*, 1995), leading to the existence of a series of subgenomic, sometimes substoichiometric DNA molecules that may replicate autonomously (Abdelnoor *et al.*, 2003; Lonsdale *et al.*, 1988; Small *et al.*, 1989). The chondrome is to a great extent composed of large, circularly permuted linear molecules with some smaller circles existing (Backert *et al.*, 1995; Bendich, 1993; Bendich, 1996; Oldenburg und Bendich, 2001).

One implication of the chondriome being organized into many morphologically discrete organelles is that there has to be fusion in order to allow for mtDNA recombination. Components of the mitochondrial fusion machinery have been identified and studied intensively in yeast and multicellular animals (Griffin *et al.*, 2006; Sesaki und Jensen, 1999; Sesaki und Jensen, 2001; Sesaki und Jensen, 2004; Sesaki *et al.*, 2003). However, no structural homologues have been identified in any plant species as yet (Okamoto und Shaw,

2005). Nevertheless there is no doubt that plant mitochondria fuse. In the last couple of years various studies have shown the fusion process microscopically using fluorescent dyes like MitoTracker and 4',6-diamidino-2-phenylindole (DAPI; (Sheahan *et al.*, 2005). In a very elegant study, Arimura *et al.* (2004) used a photoconvertible fluorescent dye called *Kaede* (Japanese for maple tree). Upon exposure to light of 350-400 nm this dye irreversibly changes from green to red. The authors were able to convert half of the mitochondria in onion cells transiently expressing Kaede to fluoresce red and then visualize the fusion between red and green organelles, which turned yellow upon doing so. They showed that after only 1-2 hours there had been enough fusion to convert all mitochondria to yellow (Arimura *et al.*, 2004).

The mitochondrial division apparatus was also studied intensively over the last ten years, mainly in *S. cerevisiae*, where at least four proteins (Dnm1p, Fis1p, Mdv1p and Caf4p) were identified to form a complex on the mitochondrial outer membrane and act together during division of the organelle (Cerveny *et al.*, 2001; Fekkes *et al.*, 2000; Griffin *et al.*, 2005; Labrousse *et al.*, 1999; Mozdy *et al.*, 2000; Okamoto und Shaw, 2005; Otsuga *et al.*, 1998; Santel und Frank, 2008; Smirnova *et al.*, 1998; Tieu und Nunnari, 2000). In higher plants homologues of some of these proteins have been identified recently. Two dynamin-like proteins with homology to yeast Dnm1p were discovered in *Arabidopsis*. Knockout of the corresponding genes, *DRP3A* or *DRP3B*, leads to an increase in mitochondrial size and a simultaneous decline in the number of mitochondria per cell, likely caused by fusion in the absence of fission (Arimura *et al.*, 2004; Arimura und Tsutsumi, 2002; Fujimoto *et al.*, 2009; Logan *et al.*, 2004; Scott *et al.*, 2006). In addition, two other members of the dynamin-like superfamily, DRP1C and DRP1E, were reported to locate to mitochondria in *Arabidopsis*, and disruption of the genes led to an increase in mitochondria with elongated morphology (Jin *et al.*, 2003). Evidence for a function in mitochondrial division still needs to be provided, though. Apart from dynamin-like proteins, a member of the plant mitochondrial division machinery, named BIGYIN, has been identified recently in *Arabidopsis* by Scott *et al.* (2006). Knockout-mutants display a phenotype very similar to that of *drp3a* or *drp3b* mutants, suggesting a role of BIGYIN in mitochondrial fission (Scott *et al.*, 2006).

Obviously, frequent inter-mitochondrial fusion ensures the chondriome of higher plants to genetically function as a discontinuous whole by allowing recombination and mixing of the mtDNA which is scattered in physically discrete organelles. A prerequisite for the frequent and essential fusion and fission processes of mitochondria is their motility. The

cytoskeleton is known to play a crucial role in normal mitochondrial morphology and distribution in many eukaryotes (Frederick und Shaw, 2007; Hollenbeck und Saxton, 2005). In plants, mitochondrial movement mainly relies on actin filaments (Romagnoli *et al.*, 2007; Van Gestel *et al.*, 2002). Homologues of mammalian and yeast proteins involved in actin-based motility have been identified in *Arabidopsis*, but whether they function in mitochondrial movement remains unclear (Kandasamy *et al.*, 2004; McKInney *et al.*, 2002).

A novel group of proteins, belonging to the family of Miro-GTPases (Fransson *et al.*, 2006; Frederick *et al.*, 2004; Guo *et al.*, 2005; Shan *et al.*, 2004), has very recently been implicated in the control of mitochondrial morphology in *Arabidopsis*. MIRO1 und MIRO2 were found to localize to mitochondria and lack of MIRO1 leads to enlarged, tube-like mitochondria (Yamaoka und Leaver, 2008).

Motility is hugely important for correct mitochondrial inheritance (Barr *et al.*, 2005) and during cell division (Logan, 2006; Sheahan *et al.*, 2004). Additionally, changes in mitochondrial morphology and distribution have been associated with processes connected to cell growth (Sheahan *et al.*, 2004), senescence (Zottini *et al.*, 2006) and various forms of induced cell death (Armstrong *et al.*, 2006; Stickens und Verbelen, 1996; Yoshinaga *et al.*, 2005) proving a pivotal role in development and physiological functions of the plant.

1.4 Organellar transcription and phage-type RNA polymerases

1.4.1 The transcription machinery of plant mitochondria

During the course of evolution mitochondria lost most of the genes that had originally been encoded by the endosymbiotic progenitor's genome either through gene transfer to the nucleus or gene loss (Huang *et al.*, 2005; Martin, 2003; Timmis *et al.*, 2004). Hence, plant mitochondrial genomes only retained a set of less than 60 genes, encoding approximately 30 proteins, up to 20 tRNAs and 3 rRNAs (Clifton *et al.*, 2004; Handa, 2003; Kubo *et al.*, 2000; Notsu *et al.*, 2002; Ogihara *et al.*, 2005; Sugiyama *et al.*, 2005; Unseld *et al.*, 1997). Interestingly, all eukaryotic mitochondria, with exception of the freshwater protist *Reclinomonas americana* (Gray *et al.*, 1998; Lang *et al.*, 1997) lost the genes encoding the bacterial-type multi-subunit RNA polymerase and instead acquired a different transcription apparatus (Gray und Lang, 1998; Hess und Börner, 1999). The protein components of this transcription machinery are encoded in the nucleus and have to be imported into the mitochondria (Gray und Lang, 1998; Weihe, 2004).

In the yeast *Saccharomyces cerevisiae*, the core catalytic entity of mitochondrial transcription, RPO41, is a single-subunit enzyme with homology to RNA polymerases of bacteriophages like T3 and T7 (Kelly *et al.*, 1986; Masters *et al.*, 1987; Matsunaga und Jaehning, 2004). The discovery of DNA sequences homologous to T3/T7 RNA polymerases throughout mitochondria of the eukaryotic lineage, including humans (Tiranti *et al.*, 1997), suggests that a phage-like enzyme was recruited early in evolution to function in mitochondrial transcription (Cermakian *et al.*, 1996; Cermakian *et al.*, 1997).

In plants, phage-type RNA polymerases are encoded by the small nuclear encoded *RpoT* gene family. Members of this family have been identified in various angiosperms such as barley (Emanuel *et al.*, 2004), wheat (Ikeda und Gray, 1999), *Nicotiana tabacum* (Hedtke *et al.*, 2002), *Nicotiana sylvestris* (Kobayashi *et al.*, 2002; Kobayashi *et al.*, 2001), *Zea mays* (Chang *et al.*, 1999; Young *et al.*, 1998), *Chenopodium album* (Weihe *et al.*, 1997), *Arabidopsis thaliana* (Hedtke *et al.*, 1997; Hedtke *et al.*, 2000) and in the moss *Physcomitrella patens* (Kabeya *et al.*, 2002; Richter *et al.*, 2002) Very recently, a single *RpoT* gene was detected and characterized in the spike-moss *Selaginella moellendorfii* (Yin *et al.*, 2009). Furthermore, homologous sequences were also detected in a gymnosperm (*Pinus taeda*; U. Richter, personal communication) and a green alga (*Chlamydomonas reinhardtii*; A. Weihe, unpublished).

In *Arabidopsis*, the *RpoT* gene family comprises three genes, encoding products that are imported into mitochondria (*RpoTm*), plastids (*RpoTp*) and dually into both organelles (*RpoTmp*; (Hedtke *et al.*, 1997; Hedtke *et al.*, 2000; Hedtke *et al.*, 1999). A similar situation is observed in *Nicotiana* species (Hedtke *et al.*, 2002; Kobayashi *et al.*, 2001). In monocots, however, so far only two *RpoT* genes could be identified, one coding for a mitochondrially targeted protein and the other for an enzyme targeted to plastids (Chang *et al.*, 1999; Emanuel *et al.*, 2004; Ikeda und Gray, 1999). In the moss *Physcomitrella patens*, two *RpoT* genes have been studied and a third gene can be found in the *Physcomitrella* genome project database (http://genomeportal.jgi-psf.org/Phypa1_1/Phypa1_1.home.html). Both characterized *RpoT* genes seem to be capable of dual targeting as a result of translation initiation at two different in-frame AUG start codons (Richter *et al.*, 2002). Richter *et al.* (2002) showed that for both *RpoT1* and *RpoT2* translation initiation at the first AUG start codon yields a product targeted to plastids, while initiation at the second start codon leads to a mitochondrially targeted enzyme (Richter *et al.*, 2002). Recently, Kabeya and Sato (2005) reported both *Physcomitrella* genes, as well as the *Arabidopsis RpoTmp* gene, to be

exclusively translated from the second AUG start codon and targeted to mitochondria *in vivo* (Kabeya und Sato, 2005).

Phylogenetic analyses suggest that the *RpoT* gene families of *Physcomitrella* and higher plants have arisen by gene duplication events dating after the separation of bryophytes and tracheophytes (Richter *et al.*, 2002).

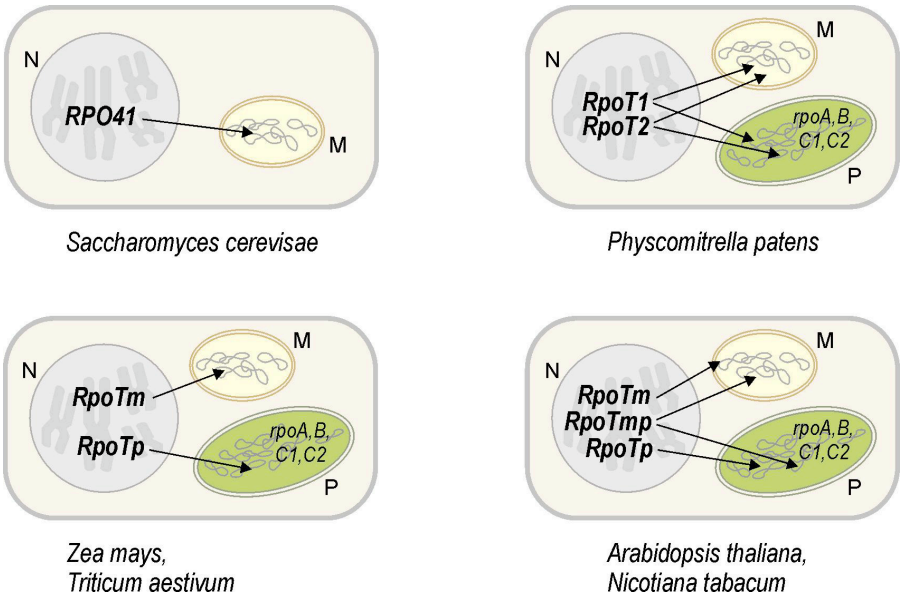


Figure 7: Nuclear genes encoding organellar phage-type RNA polymerases in different organisms.
Genes in the nucleus (N, gray) of eukaryotic organisms code for T3/T7 phage-like RNA polymerases which, following their synthesis in the cytoplasm, are imported into mitochondria (M, yellow) and plastids (P, green).

Until recently it was commonly assumed that phage and phage-like RNA polymerases descended from a common ancestor, most probably a DNA polymerase or reverse transcriptase. The origin of the first single-subunit RNA polymerase thus would have paralleled the origin of the mitochondriate eukaryotic cell (Cermakian *et al.*, 1997).

Recently, however, the discovery of cryptic prophages related to bacteriophages like T3 and T7 in several proteobacterial genomes has led to a more likely hypothesis. According to this supposition, prophages containing genes encoding phage-type enzymes, e.g. T3/T7-like RNA polymerases, were already present in the endosymbiotic predecessor of the mitochondrion. During evolution most mitochondrial genes including the prophages were transferred to the host nucleus where the latter were eventually reactivated (Filée und Forterre, 2005).

1.4.2 The transcription machinery of plastids

Unlike mitochondria, plastids have retained genes for the core subunits of a eubacteria-like RNA polymerase which was named PEP, for plastid-encoded plastid RNA polymerase (Hess und Börner, 1999; Liere und Börner, 2007; Shiina *et al.*, 2005). The enzyme is composed of three subunits which are encoded by *rpoA*, *rpoB* and *rpoC1* and *rpoC2* genes and recognizes typical eubacterial-type -10 and -35 promoter sequences (Suzuki *et al.*, 2004). Accordingly, many chloroplast genes possess conserved promoter structures containing these -10 and -35 regions (Sugiura, 1992). The core polymerase of *E. coli* needs to form a complex with distinct cofactors, called sigma-factors, to be able to recognize promoters and initiate transcription (Wösten, 1998). Sigma-factors, have also been found in several plant species, including *Arabidopsis*, maize, rice, wheat, tobacco, *Sinapis alba*, *Cyanidium caldarium* and *Physcomitrella patens*, where they are exclusively encoded in the cell nucleus (Allison, 2000; Isono *et al.*, 1997; Lysenko, 2007). Thus, even though the core enzyme is encoded by plastid genes, PEP activity is strictly under nuclear control.

Functional PEP complexes obviously exist in more than one form. In etioplasts of mustard, a fraction containing the αββ′β ′′ core subunits was isolated while mature chloroplasts possess an active complex consisting of the four core proteins plus an additional nine (Baginsky *et al.*, 1999; Loschelder *et al.*, 2004; Pfannschmidt und Link, 1997; Pfannschmidt *et al.*, 2000). Similarly, a complex of 13 polypeptides was isolated together with the PEP core subunits from tobacco chloroplasts (Suzuki *et al.*, 2004). However, the exact composition and function of the isolated proteins is still elusive. Furthermore, a membrane attached transcriptionally active chromosome (TAC) has been isolated from different organisms (Briat *et al.*, 1979; Pfalz *et al.*, 2006; Reiss und Link, 1985; Rushlow *et al.*, 1980; Suck *et al.*, 1996). It consists of several multimeric protein complexes and is able to transcribe endogenously bound DNA (Igloi und Kössel, 1992). In *Arabidopsis*, the TAC comprises at least 35 polypeptides, some of which influence plastid transcription, RNA accumulation and processing (Pfalz *et al.*, 2006).

Studies on plastid ribosome-deficient plants, such as the barley *albostrians* or the *iojap* maize mutant and various plants with directed transplastomic knockouts of *rpoA*, *rpoB* and/or *rpoC1* revealed that despite the lack of PEP the plants were viable and showed plastid transcription activity (Allison *et al.*, 1996; Bünger und Feierabend, 1980; DeSantis-Maciossek *et al.*, 1999; Han *et al.*, 1993; Hess *et al.*, 1993; Krause *et al.*, 2000; Legen *et al.*,

2002). Additionally, numerous tissue culture lines, e.g. tobacco BY-2, rice and maize BMS were shown to be PEP-deficient (Cahoon *et al.*, 2003; Kapoor *et al.*, 1997; Silhavy und Maliga, 1998; Vera und Sugiura, 1995; Vera *et al.*, 1996). While all the above mentioned plants and cell lines are photosynthetically inactive, some *Cuscuta* species lacking PEP activity even retain the ability to photosynthesize (Berg *et al.*, 2004). Taken together, this clearly indicated that there had to be another transcription activity in plastids. This activity was found to be nuclear encoded and therefore termed NEP, for nuclear-encoded plastid RNA polymerase.

In the following years, nuclear genes encoding single-subunit, phage-like RNA polymerases directed to plastids were isolated from several plant species (Chang *et al.*, 1999; Emanuel *et al.*, 2004; Hedtke *et al.*, 1997; Hedtke *et al.*, 2002; Kobayashi *et al.*, 2002; Kobayashi *et al.*, 2001; Kusumi *et al.*, 2004; Richter *et al.*, 2002). They belong to the *RpoT* gene family (see 1.3.1) and encode proteins either localized exclusively to plastids (RpoTp) or to both plastids and mitochondria (RpoTmp; see 1.3.1). RpoTp and RpoTmp most probably arose from the original mitochondrial-localized polymerase (RpoTm) through gene duplications coupled with the acquisition of new transit peptides.

All *RpoT* genes share high sequence homology with each other and the T7 RNA polymerase. The T7 bacteriophage enzyme is a 99 kDa single-polypeptide-chain protein that is able to recognize specific promoter sequences, correctly initiate transcription and catalyze transcript elongation without additional cofactors (Steitz, 2004). Plant RpoT polymerases are proteins of around 110 kDa (Hess und Börner, 1999; Lerbs-Mache, 1993). Their C-terminal amino acid sequence forming the so-called “fingers”, “palm” and “thumb” structures is highly conserved and similar to that of the T7 RNA polymerase (Chang *et al.*, 1999; Hess und Börner, 1999); see Fig. 8). This region comprises the catalytically relevant part of the enzyme (McAllister, 1993; Sousa *et al.*, 1993). Those parts of the protein contributing to promoter recognition are poorly conserved between plant and phage polymerases, possibly reflecting the divergence in promoter architecture and composition of initiating complexes (Chang *et al.*, 1999; Cheetham *et al.*, 1999; Ikeda und Gray, 1999; Jeruzalmi und Steitz, 1998; Kühn, 2005).

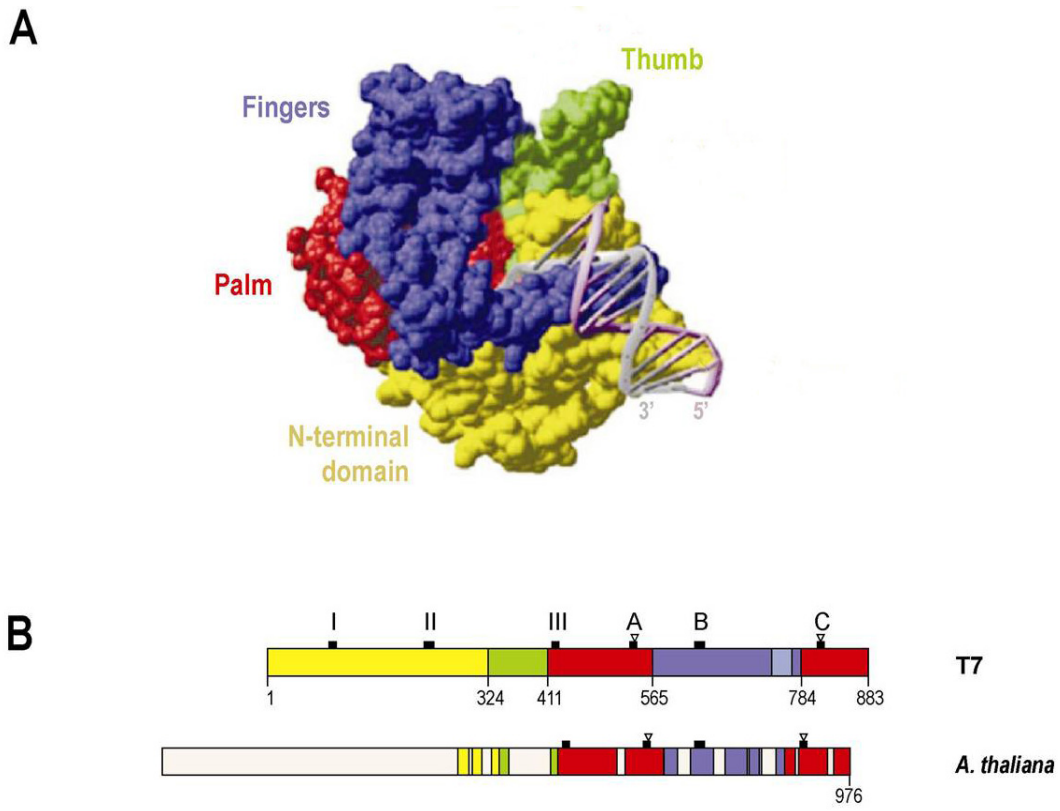


Figure 8: Structure of the T7 phage RNA polymerase and conserved regions between phage and eukaryotic phage-type enzyme.
(A) Surface representation of the T7 RNA polymerase-promoter complex structure. The N-terminal domain (yellow) and the characteristic C-terminal subdomains "palm" (red), "fingers" (blue) and "thumb" (green) are shown (after Cheetham *et al.*, 1999). (B) Amino acid sequence organization of T7 phage RNA polymerase domains and homologous amino acid sequence regions of an organellar phage-type RNA polymerase, based on a sequence comparison by Hedtke *et al.*, 1998. Color scheme is the same as in (A). *Arabidopsis thaliana* RpoTm is shown as an example of mitochondrial and plastidial enzymes of land plants which do not greatly differ in their organization. Black squares mark the positions of the motifs A, B, C and III that are important for RNA polymerase function and conserved in all enzymes. Motifs I and II are important for promoter recognition in T7 phages and are not present in eukaryotic polymerases. Open triangles denote the invariant residues Asp537 and Asp812 acting as ligands to two catalytic Mg^{2+} ions at the active site (Woody *et al.*, 1996).

1.4.3 Regulation of organellar gene expression by phage-type RNA polymerases

The significance of the existence of two or even three different RNA polymerases in higher plant plastids remains largely elusive. Promoter studies revealed three generally different types of plastid genes: PEP-transcribed genes, NEP-transcribed genes and genes that possess both PEP and NEP promoters (DeSantis-Maciossek *et al.*, 1999; Hajdukiewicz *et al.*, 1997; Hess und Börner, 1999; Hübschmann und Börner, 1998; Liere und Maliga, 2001; Maliga, 1998). Genes that are transcribed by PEP mostly encode proteins involved in photosynthesis-related functions, whereas only a few genes that constitute the plastid genetic machinery, including the subunits of PEP are solely transcribed by NEP

(Hajdukiewicz *et al.*, 1997). Most genes involved in housekeeping, such as transcription and translation, possess both types of promoters. The NEP promoters are mostly silent, though, in chloroplasts (Liere und Maliga, 2001). These data suggest a sequential action of the different polymerases. NEP is supposed to be active very early in development and in non-green tissue to transcribe the subunits of PEP and initiate its function, whereas the latter is predominating in mature, photosynthetically active tissues (Hess und Börner, 1999; Liere und Maliga, 2001).

However, recent studies prove the situation to be more complex. NEP activity is found in all kinds of plastids, even in mature chloroplasts (Hajdukiewicz *et al.*, 1997). Furthermore, analyses using transplastomic tobacco mutants that lack PEP due to a knockout of one of the *rpo* genes revealed that these plants are able to transcribe all plastid genes including those having only PEP promoters (Krause *et al.*, 2002; Legen *et al.*, 2002). In wildtype plants, however, NEP does not seem to transcribe PEP genes, because PEP-transcript 5'-ends generally map to canonical PEP-promoters (Cahoon *et al.*, 2006). Thus, the roles of the different plastidial RNA polymerases seem to overlap to a certain degree and their division of labor obviously is highly complex.

The situation is even more complex in dicotyledonous plants, where a third phage-like RNA polymerase (RpoTmp) comes into play. This enzyme is dually targeted to both plastids and mitochondria (Hedtke *et al.*, 2000). Therefore, both RpoTp and RpoTmp can be considered to be part of the NEP activity. Evidence for a role of RpoTp in plastid transcription comes from analyses of RpoTp-overexpressing tobacco pants. The study revealed an enhanced transcription from distinct NEP promoters (Liere *et al.*, 2004). Knockout of *RpoTp* in *Arabidopsis* was shown to lead to altered accumulation of NEP-generated transcripts, such as *rpoB*, *clpP* and *accD*, further suggesting a role of RpoTp in NEP activity (Hricová *et al.*, 2006). Additionally, the ability of RpoTp to recognize distinct NEP promoters *in vitro* was demonstrated recently (Kühn *et al.*, 2007). In the same study, RpoTmp was not able to bind NEP promoters *in vitro* (Kühn *et al.*, 2007) and in an earlier report Kabeya and Sato (2005) suggested RpoTmp to be solely targeted to mitochondria *in vivo* (Kabeya und Sato, 2005). Nevertheless there are hints for an involvement of RpoTmp in NEP activity. Azevedo *et al.* (2006) found a spinach homolog to be localized exclusively to plastids but not to mitochondria (Azevedo *et al.*, 2006). In *Arabidopsis*, knockout of *RpoTmp* led to defects only in plastid gene expression together with delayed greening as well as leaf and root growth phenotypes in young seedlings. Later in their development the plants grew normally. Mitochondrial gene expression was not affected (Baba *et al.*, 2004).

Supporting evidence for a role of RpoTmp especially in plastids of young seedlings comes from a study of Emanuel *et al.* (2005). They did not find *RpoTp* expression until seven days after germination, while the other *RpoT* transcripts accumulated much earlier (Emanuel *et al.*, 2006). Direct evidence of a distinct function of RpoTmp in plastidial transcription came from a study of Courtois *et al.* (2007). The authors found that RpoTmp specifically transcribes the *rrn* operon from the PC-promoter during seed imbibition in *Arabidopsis* (Courtois *et al.*, 2007). In a more recent study, RpoTmp was further suggested to transcribe non-consensus (type-II) promoters in *Arabidopsis* chloroplasts (Swiatecka-Hagenbruch *et al.*, 2008). Using quantitative real-time PCR, histochemical β-glucuronidase (GUS) assays and *in situ* hybridization, Emanuel *et al.* (2005) found an overlapping expression pattern of *RpoTm* and *RpoTmp*, and a completely different pattern of *RpoTp* expression in *Arabidopsis*. They therefore suggest that RpoTm and RpoTmp recognize different types of mitochondrial promoters. Furthermore they propose RpoTp to be the major NEP transcription activity in green tissue, while RpoTmp functions mainly in non-green plastids (Emanuel *et al.*, 2006).

Expression of the *RpoT* genes is regulated on different levels. It was shown that in *Arabidopsis*, transcript levels of *RpoTm* are significantly higher in roots compared to leaves, while the opposite is true for *RpoTp* (Baba *et al.*, 2004). Generally, *RpoTm* transcripts seem to accumulate most in mitochondria-rich, meristematic tissue, such as root tips and companion cells of the phloem, whereas transcript levels of *RpoTp* are highest in green tissue like parenchyme cells, the primary cortex of the stem and sepals of buds and flowers (Emanuel *et al.*, 2006). Interestingly, transcripts of all *RpoT* genes accumulated to highest levels in flowers, while overall expression in all other organs was very low. *RpoTm* transcripts were most abundant in all tissues except mature leaf cells, where *RpoTp* transcripts showed highest accumulation (Emanuel *et al.*, 2006).

RpoT gene expression was also analyzed in several monocotyledonous plants. Leaves of grasses show a developmental gradient from the base, comprising the youngest cells, to the tip, where the oldest cells are found (Baumgartner *et al.*, 1993; Baumgartner *et al.*, 1989; Dale, 1972; Dean und Leech, 1982; Leech *et al.*, 1973; Thompson *et al.*, 1998). In maize leaves *RpoTp* transcript levels were found to be high in the basal part and increasing progressively along the leaf, reaching a peak near the mid-point of the leaf and then decreasing markedly to the tip. Protein abundance of both RpoTp and RpoTm was highest near the base and tapered off along the length of the leaf (Cahoon *et al.*, 2004). Very similar transcript and protein patterns were detected in stems and leaves of rice (Kusumi *et al.*,

2004). In the same study, analyses of a chloroplast-deficient rice mutant suggested NEP activity to be affected by the developmental state of chloroplasts (Kusumi *et al.*, 2004). *RpoTp* transcripts in barley leaves were most abundant in the leaf base, then declined and reached a second peak about 1 cm above the base. Along the remainder of the leaf, transcript levels remained constant (Emanuel *et al.*, 2004). This pattern most likely reflects the enzyme's activity as transcript accumulation of NEP-transcribed genes (*clpP*, *rpl2* and *rpoB*) closely followed the transcription of *RpoTp*, thereby proving RpoTp and NEP to be identical (Emanuel *et al.*, 2004). *RpoTm* transcript abundance in barley leaves was highest in the basal part, then declining rapidly within the first centimeter and stayed constantly low until the tip of the leaf. A huge difference of about tenfold was detected between the maximum at the base and the minimum in mature chloroplasts (Emanuel *et al.*, 2004).

In white, chloroplast-deficient leaves of the barley *albostrians* mutant, *RpoTp* transcript levels were comparable to green leaves in the basal-most part, but increased quickly and stayed more or less constant, slightly declining towards the tip. In the mature parts of the leaves, transcript abundance was up to eightfold higher compared to green leaves. Transcript levels of *RpoTm* were also increased significantly in white leaves.

Considering all these results, *RpoT* gene expression seems to be regulated by various factors, such as tissue type, age of the cells and especially the developmental status of plastids. Interorganellar crosstalk and communication between the organelles and the nucleus obviously plays a major role in the regulation of plastid and mitochondrial RNA polymerases.

1.5 Aim of this work

Although it is commonly accepted that RpoTp and RpoTmp are the RNA polymerases responsible for NEP (nuclear encoded plastid RNA polymerase) activity in higher plants, not much is known about regulation of *RpoT* gene expression. To date differential developmental and tissue-specific abundance of *RpoT* transcripts has been shown for *Arabidopsis* and barley (Baba *et al.*, 2004; Emanuel *et al.*, 2006; Emanuel *et al.*, 2004). Since plastids are the sites of photosynthesis, it is not surprising that the expression of a number of plastid and nuclear encoded genes involved in photosynthesis is regulated in a light-dependent manner (Christopher und Mullet, 1994; Granlund *et al.*, 2009; Link, 1996; Mayfield *et al.*, 1995; Mochizuki *et al.*, 2004; Pfannschmidt *et al.*, 1999; Pfannschmidt *et al.*, 1999; Rapp *et al.*, 1992; Terzaghi und Cashmore, 1995; Thompson und White, 1991;

Tsunoyama *et al.*, 2004; Tsunoyama *et al.*, 2002).

NEP transcribes not only important plastid housekeeping genes, but also the *rpoB* operon, encoding major subunits of PEP which is accountable for the transcription of most photosynthesis genes in plastids (for reviews see (Hess und Börner, 1999; Liere und Maliga, 2001). In the present study quantitative real-time PCR analyses will be applied to learn more about light-induced accumulation of *RpoT* gene transcripts. To this end, transcript accumulation in wild-type plants will be analyzed in different developmental stages. To further get an insight into which photoreceptors and pathways may be involved in light-induced transcription of the *RpoT* genes, wild-type seedlings will be analyzed under different light qualities. Additionally, different photoreceptor mutants will be investigated.

Organellar metabolism and function in higher plants involves a complex network of plastids, mitochondria and the nucleus. Concerted transcription of organellar genes thus requires distinct communication of all compartments in a cell. To learn more about this communication, transcription of organellar genes will be analyzed in normal, green and spectinomycin-treated, white *Arabidopsis* seedlings by quantitative real-time PCR. It has been shown that copy numbers of mitochondrial genes may change under certain circumstances (Fujie *et al.*, 1993; Kuroiwa *et al.*, 1992; Takanashi *et al.*, 2006), but the physiological impact of this phenomenon remains elusive. In order to learn more about that interesting regulation, mitochondrial gene copy numbers will be analyzed during leaf development and in green versus white *Arabidopsis* plants.

2 Materials and Methods

2.1 Materials

Chemicals and biochemicals used in this study were generally purchased from Biozym, Roth, ICN Biomedical, Merck, Serva, Sigma-Aldrich and Qiagen, unless specified otherwise. Ultrapure water was obtained from a USF Purelab Plus system. Sterilization of solutions, buffers and hardware, as well as inactivation of genetically modified material was carried out in the Varioklav 75 S steam sterilizer (Thermo Scientific) at 120°C and 55 kPa for 20 minutes.

2.1.1 Providers

Applied Biosystems	Applied Biosystems, Weiterstadt, Germany
Ambion	Ambion, Inc., Austin, TX, USA
Amersham Biosciences	Amersham Biosciences Europe GmbH, Freiburg, Germany
Biometra	Biometra GmbH, Göttingen, Germany
Bio-Rad	Bio-Rad Laboratories, Richmond, VA, USA
Biozym	Biozym Diagnostik GmbH, Hameln, Germany
Braun	Braun GmbH, Kronberg, Germany
Calbiochem	Calbiochem Merck Biosciences GmbH, Schwalbach, Germany
DuPont	DuPont de Nemours GmbH, Bad Homburg, Germany
Epicentre	Epicentre Biotechnologies, Madison, WI, USA
Eurogentec	Eurogentec, Seraing, Belgium
Fermentas	Fermentas GmbH, St. Leon-Rot, Germany
GE Healthcare	GE Healthcare Europe GmbH, Freiburg, Germany
Heraeus	Heraeus, Hanau, Germany
Invitrogen	Invitrogen GmbH, Karlsruhe, Germany
Jenoptik	Jenoptik L.O.S. GmbH, Jena, Germany
Macherey-Nagel	Macherey-Nagel, Düren, Germany
Metabion	metabion international AG, Martinsried, Germany
Millipore	Millipore Corp., Bedford, USA
Nalgene	Nalgene®Labware, Rochester, NY, USA
Operon	Operon Biotechnologies GmbH, Köln, Germany
peqLab	peqLab Biotechnologie GmbH, Erlangen, Germany
Perkin Elmer	Perkin Elmer LAS (Germany) GmbH, Rodgau, Germany
Pierce	Pierce, Rockford, IL, USA
Promega	Promega Corp., Madison, WI, USA
Qiagen	Qiagen, Hilden, Germany
Roche	Roche Diagnostics GmbH, Mannheim, Germany
Roth	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Serva	Serva Feinbiochemika, Heidelberg, Germany
Sorvall	Kendro Laboratory Products GmbH, Langenselbold, Germany
Sigma	Sigma Chemical Company, St. Luis, MO, USA
Stratagene	Stratagene, La Jolla, CA, USA
Thermo Scientific	Thermo Scientific LED GmbH, Langenselbold, Germany
USF	USF, Seral Reinstwassersysteme GmbH, Germany
Whatman	Whatman Paper, Maidstone, UK
Zeiss	Carl Zeiss MicroImaging GmbH, Jena, Germany

2.1.2 Plant material

Arabidopsis thaliana wild type plants were grown from seeds of the ecotype Columbia. Seeds of the different photoreceptor mutants (Table 1) were kindly provided by Prof. Hellmann (Freie Universität Berlin) and Prof. Batschauer (Philipps-Universität Marburg).

Tab. 1: Employed mutant plants

name	mutation	mutant denotation	ecotype background
phyA	knockout of the gene *PHYA*, leading to plants lacking the photoreceptor phytochrome A	*phyA*	Ler
phyB	knockout of the gene *PHYB*, leading to plants lacking the photoreceptor phytochrome B	*phyB*	Ler
phyA/phyB	knockout of the genes *PHYA* and *PHYB*, leading to plants lacking the photoreceptors phytochrome A and B	*phyA-201/phyB-5*	Ler
cry1	knockout of the gene *CRY1*, leading to plants lacking the photoreceptor cryptochrome 1	*hy4-1*	Ler
cry2	knockout of the gene *CRY2*, leading to plants lacking the photoreceptor cryptochrome 2	*fha-1*	Ler
cry1/cry2	knockout of the genes *CRY1* and *CRY2*, leading to plants lacking the photoreceptors cryptochrome 1 and 2	*hy4-2.23N/fha-1*	Ler
hy5	knockout of the gene *HY5*, leading to plants lacking the transcription factor hy5	*hy5*	Ler

2.1.3 Bacterial strains

Recombinant plasmids were propagated in *E. coli* Top10 cells (Invitrogen).

2.1.4 Oligonucleotides

Oligonucleotides were provided by Sigma-Genosys or Operon. Annealing temperatures of primers were calculated and verified using the Oligo Calculation Tool from GenScript Corporation (http://www.genscript.com/cgi-bin/tolls/primer_calculation). Sequences of oligonucleotides are denoted in the respective chapters.

2.1.5 Software

Primers for quantitative real-time PCR were designed using the ProbeFinder Software of the Universal ProbeLibrary Assay Design Center (Roche Applied Science, https://www.roche-applied-science.com/sis/rtpcr/upl) or the Primer-Express® software v2.0 (Applied Biosystems). Database and homology searches in public sequence databases at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) were conducted using the BLAST algorithm, version 2.2.16 at http://www.ncbi.nlm.nih.gov/BLAST (Altschul *et al.*, 1990; Altschul *et al.*, 1997). Drafting of text and graphics and image processing were carried out using Microsoft® Office Word 2007, Microsoft® Office Excel 2007, Microsoft® Office Power Point 2007 and Adobe® Photoshop, version 9.0.

2.2 Methods

2.2.1 Plant growth

Plants for leaf development and respiration rate analyses

Arabidopsis thaliana (ecotype Columbia) seeds were sown on SEA medium under short-day conditions (8h light / 16h darkness) at 20°C; cotyledons were harvested after five and ten days.

For leaf samples, seeds were sown on vermiculite/soil-mix (1:4) under short-day conditions (8h light / 16h darkness) at 20°C. After fourteen days, seedlings were shifted to long-day conditions (16h light / 8h darkness). Light intensity was set at ~ 150 µmol photons * s^{-1} * m^{-2}. Age determination and harvesting of the leaves were carried out as described by R. Zoschke (Zoschke, 2005; Zoschke *et al.*, 2007).

Comparison of green and white plants

Surface-sterilized *Arabidopsis thaliana* (ecotype Columbia) seeds were sown on sterilized SEA medium containing sucrose (10 g / L). Germinating seeds on SEA medium containing spectinomycin (500 mg / L) generated chlorophyll-deficient plants. Plants were grown under long-day conditions (see above) at 23°C. After ten and twenty days, whole seedlings were harvested. Light intensity was set at ~ 150 µmol photons * s^{-1} * m^{-2}.

Seedlings for light induction analyses and photoreceptor mutants

Surface-sterilized *Arabidopsis thaliana* (ecotype Columbia) seeds were sown on sterilized SEA medium containing sucrose (10 g / L). Plants were grown in complete darkness at 23°C. After seven days, part of the seedlings was harvested directly as dark controls. The remaining etiolated seedlings were put into the light and harvested after one, four, six, twelve and twenty-four hours. Light intensity was set at ~ 150 µmol photons * s^{-1} * m^{-2}.

Plants for light induction experiments

Arabidopsis thaliana (ecotype Columbia) seeds were sown on vermiculite/soil-mix (1:4). Plants were grown in a 12h light / 12h darkness regime at 20°C. After 19 and 63 days they were transferred to complete darkness for 72h. Subsequently, leaf samples from part of the plants were harvested directly as dark controls. The remaining plants were exposed to light for one, six and twelve hours. Light intensity was set at ~ 150 µmol photons * s^{-1} * m^{-2}

Different light qualities

Surface-sterilized *Arabidopsis thaliana* (ecotype Columbia) seeds were sown on sterilized SEA medium containing sucrose (10 g / L). Plants were grown in complete darkness at 23°C. After seven days, part of the seedlings was harvested directly as dark controls. The remaining etiolated seedlings were put into light of a certain wavelength and harvested after one, four, six, twelve and twenty-four hours. Different light regimes were

achieved by placing LED arrays in a darkened chamber. Three different LED arrays were used producing monochromatic red (631 nm), blue (470 nm) and green (525 nm) light.
Plants for circadian clock experiments

Arabidopsis thaliana (ecotype Columbia) seeds were sown on vermiculite/soil-mix (1:4). Plants were grown in a 12h light / 12h darkness regime at 20°C. After three weeks they were exposed to constant light. Harvesting of leave samples began 24 hours after the onset of constant light and samples were subsequently taken every 4 hours for a total of 48 hours. Light intensity was set at ~ 150 µmol photons * s^{-1} * m^{-2}

SEA medium:	0.44 % (w/v) MS basal medium; 0.05 % (w/v) MES in ultrapure water; pH 5.7 with KOH; 1.5 % (w/v) agar for plant cell cultures

2.2.2 Surface sterilization of Arabidopsis seeds

Seeds were incubated in sterilization solution and shaken gently. After seven minutes they were spun down in a microcentrifuge and the supernatant was aspired. Seeds were then washed in sterile ultrapure water and spun down again. This was repeated five times. After the last washing step seeds were transferred to a petri dish with sterilized SEA medium.

sterilization solution:	20 ml DanKlorix (Colgate-Palmolive GmbH), 40 ml ultrapure water, 2.5 ml N-lauryl-sarcosine-sol. (20%)

2.2.3 Isolation of nucleic acids

2.2.3.1 Isolation of genomic DNA

Genomic DNA from *Arabidopsis thaliana* samples was isolated following the CTAB protocol (Murray und Thompson, 1980) and subsequently treated with RNaseA/T1 mix (Fermentas). Afterwards the enzyme was removed by phenol/chloroform extraction and the DNA was precipitated using 2.5 vol ice cold 96% EtOH and 1/10 vol 3M sodium acetate at -20°C overnight or at -70°C for at least one hour.

The concentration of the DNA was determined spectrophotometrically using the Nanodrop® ND-1000 system (peqLab). Quality of DNA was checked by agarose gel electrophoresis (see 2.2.5).

2.2.3.2 Isolation of total RNA

Total RNA from *Arabidopsis* samples was isolated using the RNeasy® Plant Mini Kit (Qiagen) with Buffer RLT according to the manufacturer‘s protocol. RNA quality was controlled by denaturing agarose gel electrophoresis (see 2.2.6) and concentrations were quantified spectrophotometrically (see above).

2.2.4 Gel electrophoresis of nucleic acids

2.2.4.1 Preparative and analytical agarose gel electrophoresis of DNA

Analysis of PCR products and gDNA quality was performed by agarose gel electrophoresis. DNA samples were mixed with DNA loading dye and separated according to their molecular size. Agarose gels of 0.8 – 2.0 % (w/v) including 0.2 µg/ml EtBr were used in 1x TAE running buffer (Sambrook und Russel, 2001). The impressed voltage was set at 5 - 10 V/cm in horizontal electrophoresis chambers (PerfectBlue gel system Mini S or Mini L, peqLab). GeneRuler™ 1 kb DNA Ladder, 100 bp Plus DNA Ladder or 50 bp DNA Ladder (Fermentas) were used as molecular weight markers. DNA bands were subsequently visualized under UV-light excitation in the Gel Doc XR System (Bio-Rad).

DNA fragments were separated by preparative agarose gel electrophoresis. For this purpose, DNA bands were excised from the gel and subsequently eluated and purified using the QIAquick® Gel Extraction Kit and the QIAquick® PCR Purification Kit (Qiagen), respectively, according to the manufacturer's protocols.

1x TAE	40 mM Tris-acetate, pH 8.0; 1 mM EDTA
DNA loading dye	50 % (v/v) glycerol; 1 mM EDTA, pH 8.0; 0.05 % (w/v) bromophenol blue; 0.05 % (w/v) xylene cyanol

2.2.4.2 Analytical agarose gel electrophoresis of RNA

RNA samples were mixed with RNA loading dye, denatured at 95°C for 10 min, incubated on ice for 5 min, and subsequently separated in a 1 % (w/v) agarose gel containing $^{1}/_{40}$ vol formaldehyde in 1x MEN running buffer. The voltage was set at 2.5 - 5 V/cm. RNA bands were subsequently visualized under UV-light excitation in the Gel Doc XR System (Bio-Rad).

10x MEN	200 mM MOPS; 50 mM NaAc; 10 mM EDTA; pH 7.0 with NaOH
RNA loading dye	1 ml formamide; 350 µl formaldehyde, 200 µl 10x MEN; 400 µl glycerol; 5 µl 0.5 M EDTA, pH 8.0; 10 µl 10 mg/ml EtBr; 2 mg bromophenol blue; 2 mg xylene cyanol; ultrapure water ad 2 ml

2.2.5 Reverse transcription of total RNA

The QuantiTect® Reverse Transcription Kit (Qiagen) was used to eliminate remaining genomic DNA from the RNA samples and subsequently reverse transcribe the RNA according to the manufacturer's protocol.

2.2.6 Quantitative real-time PCR

2.2.6.1 Quantitative real-time PCR using Sybr® Green

Primer pairs for quantitative real-time PCR of genomic DNA samples were designed to yield amplification products of 70 – 100 bp. The PCR reactions were carried out in a 7500 Real-Time PCR System (Applied Biosystems) using the *Power* Sybr® Green PCR Master Mix (Applied Biosystems) or the qPCR™ Mastermix Plus for Sybr® Green I – Low Rox (Eurogentec). Each reaction contained 0.1 ng gDNA or 50 ng cDNA and 1 µM of each primer. The cycle protocol consisted of an initial step at 95°C for 10 min to activate the polymerase, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. To verify the specificity of gDNA amplification products a dissociation curve was added for each of the 96 wells by subjecting the samples to a heat denaturation over a temperature gradient from 60°C to 95°C at 0.03°C/s. To verify removal of genomic DNA from cDNA samples, a negative control (without addition of reverse transcriptase) was included for each reverse transcribed RNA sample. Each of the biological and technical replicates was analyzed in triplicates per experiment. No-template controls (NTC) were included for each primer pair. Data were analyzed using the Sequence Detection Software v1.4 (Applied Biosystems). All quantitations were normalized to the amount of the nuclear encoded single-copy gene *RpoTp* (gDNA) or the *UBQ11* transcript level (cDNA) as internal standards using the ΔC_T method ($2^{(-\Delta C_T)}$ = relative amount of gene copies; $\Delta C_T = C_T^{\text{target}} - C_T^{\text{internal standard}}$).

2.2.6.2 Quantitative real-time PCR using molecular probes

Primer pairs for quantitative real-time PCR of cDNA samples were designed to yield amplification products of 70 – 100 bp. The PCR reactions were carried out in a 7500 Real-Time PCR System (Applied Biosystems) using the *TaqMan*® Fast Universal PCR Master Mix (Applied Biosystems) or the qPCR™ MasterMix Plus Low Rox (Eurogentec). Each reaction contained 50 ng cDNA, 1 µM of each primer and 100 nm of the particular probe. The cycle protocol consisted of an initial step at 95°C for 10 min to activate the polymerase, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. To verify removal of genomic DNA from cDNA samples, a negative control (without addition of reverse transcriptase) was included for each reverse transcribed RNA sample. Each of the biological and technical replicates was analyzed in triplicates per experiment. No-template controls (NTC) were included for each primer pair. Data were analyzed using the Sequence Detection Software v1.4 (Applied Biosystems). All cDNA quantitations were normalized to the amount of *UBQ11* transcripts as internal standard using the ΔC_T method ($2^{(-\Delta C_T)}$ = relative amount of transcripts; $\Delta C_T = C_T^{\text{target}} - C_T^{\text{internal standard}}$).

Tab. 2: Primers used in quantitative real-time PCR analyses

primer denotation	nucleotide sequence (5'→3')	T_m [°C]
5'*AtRpoTm*_PL	ACAGAAATTGCGGCTAGGG	60.2
3'*AtRpoTm*_PL	GGCATATGTGGCATTTGGA	60.3
5'*AtRpoTmp*_PL	CGATGCCATTGAACAAGAGAT	60.1
3'*AtRpoTmp*_PL	TGTTCCTTCATAGAAGTTTCATTTTC	59.2
5'*AtRpoTp*_PL	TTGCAGAAGTGAAAGACATCTGA	60.0
3'*AtRpoTp*_PL	ATCGACCGTGTTACCCTCTC	59.0
5'*AtUBQ*_PL	CTTATCTTCGCCGGAAAGC	59.9
3'*AtUBQ*_PL	GAGGGTGGATTCCTTCTGG	59.4
5'*AtRpoTp*	TGGAAGCCGTCTGCTAGAACTA	61.4
3'*AtRpoTp*	TGTCTGAATGCAGGTCGAAAC	61.2
5'*q-atp1*	CTTAGAAAGAGCGGCTAAACGA	60.2
3'*q-atp1*	GGGAATATAGGCCGATACGTCT	60.5
5'*q-rps4*	CCCATCACAGAGATGCACAGA	62.3
3'*q-rps4*	GGAGACGAAGCGGAATAACGT	62.7
5'*q-cox1*	GCCATGATCAGTATTGGTGTCTT	60.3
3'*q-cox1*	CTACGTCTAAGCCCACAGTAAACA	59.8
5'*q-nad6*	AGGATGTATTCCGACGAAATGC	62.4
3'*q-nad6*	CGTGAGTGGGTCAGTCGTCC	64.2
253 5'*At-clpP*	CCTATTGGCGTTCCAAAAGTA	59.1
254 3'*At-clpP*	TCGCACTATATGTCAACCCAAG	60.0
*5'At-rrn16-PL69_*	TGAACAGACTGCCGGTGATA	60.4
*3'At-rrn16-PL69_*	AAGGGGCATGATGACTTGAC	60.4

2.2.7 Construction of a vector to test PCR efficiencies

To compare the efficiency of the different DNA assays employed for quantitative real-time PCR, fragments of the analyzed genes including each amplicon region were cloned into pBluescript® II SK (+) (Fermentas) so that it contained one copy of each gene fragment. Primers were designed using the Primer-Express® software v2.0 (Applied Biosystems). Primer sequences are denoted in Table 3. Vector DNA was then used as a template for real-time PCR. The ratio between two genes was calculated as $2^{(-\Delta C_T)}$ ($\Delta C_T = C_T^{\text{gene1}} - C_T^{\text{gene2}}$).

Tab. 3: Primers used to amplify gene fragments of four mitochondrial genes

primer denotation	nucleotide sequence (5'→3')	T_m [°C]
5'pBS-atp1	cgactcgagTTGTAGCAGCCACCGCTTC	71.9
3'pBS-atp1	tcgaagcttTTCAACTGAGCGGCAGACC	69.0
5'pBS-rps4	cgagatatcAATACAACGCCGCATTCTCC	67.5
3'pBS-rps4	tcgctgcagGCGGTTGATGCAGTAATTTGC	70.1
5'pBS-cox1	cgacccgggTCTTCGGTCATCCAGAGGTGT	74.2
3'pBS-cox1	tcgcccgggGCAAAAACGGCTCCCATAGA	73.1
5'pBS-nad6	cgagggcccGCCCTGCTTTGGTCTCTGG	72.8
3'pBS-nad6	tcggggcccCCTTTCCGGCATAAACCGT	72.8

2.2.8 Amplification of DNA using PCR

Approximately 100 ng of *Arabidopsis thaliana* gDNA were used for amplification. A 50 µl reaction in Taq reaction buffer (Qiagen) contained 2.5 U Taq DNA polymerase, 10 pmol of each forward and reverse primer and 10 nmol of each dATP, dCTP, dGTP and dTTP. PCR amplification was carried out in a Peltier Thermal Cycler PTC-200 (Biozym) using the following protocol: 95°C for 3 min followed by 40 cycles of 95°C for 30 sec, primer specific annealing temperature for 30 sec and 72°C for 1 min, and a final elongation step at 72°C for 10 min.

Verification of successful ligation of DNA fragments was done by colony PCR. A 20 µl reaction contained cells from the bacterial colony in Taq reaction buffer with 1 U Taq DNA polymerase, 5 pmol of each forward and reverse primer and 5 nmol of each dNTP.

Analysis of PCR products was done on agarose gels (see 2.2.5).

2.2.9 Cloning and sequencing

2.2.9.1 Restriction and ligation of DNA molecules

Digestion of DNA by restriction endonucleases and ligation procedures were carried out following the standard protocols by Sambrook and Russel (2001). Restriction endonucleases and pBluescript® II SK (+) vectors were supplied by Fermentas. Reactions were carried out in buffers and at temperatures according to the manufacturer's recommendations.

2.2.9.2 Transformation of E. coli

3 µl of a standard ligation reaction were added to 50 µl chemically competent *E. coli* Top10 cells (Invitrogen). Cells were then incubated on ice for 15 min and subsequently subjected to a heatshock at 42°C for 30 sec. After another short incubation on ice and addition of ~ 300 µl hand warm LB medium, samples were shaken at 220 rpm and 37°C for 1 hour. Positive transformants were selected on LB agar plates containing antibiotics according to the plasmid encoded resistance.

LB medium:	1 % (w/v) tryptone; 0.5 % (w/v) yeast extract; 1 % (w/v) NaCl in ultrapure water
LB agar:	LB medium with 1.5 % (w/v) agar

2.2.9.3 Preparation of plasmid DNA

Plasmid DNA minipreparation was performed following the standard protocol of alkaline lysis (Sambrook und Russel, 2001).

Buffer 1:	50 mM Tris/HCl pH 8.0; 10 mM EDTA; 100 µg/ml RNaseA
Buffer 2:	200 mM NaOH; 1 % (w/v) SDS
Buffer 3:	3 M NaAc pH 5.0

2.2.9.4 Sequencing

Sequencing of plasmid DNA or PCR fragments was performed using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's temperature protocol. One reaction contained each 10 pmol of forward and reverse primers (Tab. 4). *Cycle Sequencing*, as well as sample purification and analysis using the automatic ABI 377 DNA Sequencer (Applied Biosystems) were accomplished by Antje Sonntag and Dr. Martin Meixner (SMB).

Tab. 4: Primers used for DNA sequencing of plasmids

primer denotation	nucleotide sequence (5'→3')	T_m [°C]
T3-seq	GCAATTAACCCTCACTAAAGGG	58.3°C
T7-seq	GTAATACGACTCACTATAGGGC	58.4°C

2.2.10 Flow-cytometric analysis of nuclear endopolyploidy

Using at least three independent leaf samples, preparation, flow cytometric measurements and sorting of nuclear suspensions were carried out with help of Dr. Jörg Fuchs (IPK Gatersleben) as described by Barow and Meister (Barow und Meister, 2003). A FACStar™ PLUS flow cytometer employing an INNOVA 90C argon-ionic laser (Becton-Dickinson) was used for the experiment; data were analyzed with the Cell Quest v3.3 software (Becton-Dickinson). The C-values of about 10,000 nuclei were measured per leaf sample. The mean C-value was determined as a weighted average by calculating $[(2 * n_{2C}) + (4 * n_{4C}) + (8 * n_{8C})\ldots] / [n_{2C} + n_{4C} + n_{8C}\ldots]$ where n is the number of nuclei and C is the endopolyploidy level (2C, 4C, 8C…).

2.2.11 Measurement of O2-consumption in Arabidopsis leaves and cotyledons

Leaf discs of approximately 8 mm diameter or single cotyledons were prepared and transferred into the temperature controlled measuring chamber of an oxygen electrode (Hansatech, Kings Lynn, Norfolk, UK) containing 1 ml buffer solution. The temperature was kept constant at 25°C in darkness, to measure O_2-consumption of the discs. Each measurement took ~ 30 min and all measurements were terminated before a 50% depletion of oxygen to avoid O_2 limitation.

Buffer solution: 10 mM MES-KOH, pH 6.5, equilibrated in ambient air

2.2.12 Detection of proteins by Western blotting

2.2.12.1 Protein extraction from Arabidopsis leaves and seedlings

To extract total protein from plant tissues, ~ 200 mg plant material were ground in liquid nitrogen. The powder was subsequently resolved in 500 µl extraction buffer, vortexed for 20 sec and incubated on ice for 10 min. Samples were then spun down in a microcentrifuge for 5 min at 4°C two times and the supernatant was transferred to a new tube. To precipitate the proteins, 4 vol acetone were added and the sample was incubated at -20°C for at least 20 min. Finally, proteins were spun down in a centrifuge at 12,000 rpm and 4°C for 15 min and

resolved in Urea-buffer. Protein concentrations were compared to a BSA standard as described by Bradford (Bradford, 1976) using the Bio-Rad Protein Assay.

extraction buffer:	50 mM sodium phosphate pH 7.0; 1 mM EDTA pH 8.0; 1 % Triton X-100; 10 mM ß-mercaptoethanol; 2mM PMSF (50 mg/ml)
Urea-Buffer	8 M Urea; 100 mM sodium phosphate; 10 mM Tris/HCl

2.2.12.2 SDS polyacrylamide gel electrophoresis

Protein samples were separated electrophoretically according to Laemmli (Laemmli, 1970) in BioRad Mini Protean vertical gel systems. Electrophoresis was allowed to proceed at 30 mA for 1 – 1.5h. Fermentas PageRuler™ Prestained Protein Ladder #SM0671 was run alongside samples as a molecular weight marker and to visualize successful Western blot transfers in later stages (see 2.2.17).

Following electrophoresis gels were Coomassie-stained with the GelCode® Blue Stain Reagent (Pierce) or subjected to Western blotting (see 2.2.17).

5x sample buffer:	0.32 M Tris/HCl pH 6.8; 0.1 M EDTA; 0.4 M DTT; 8 % (w/v) SDS; 4 % (v/v) glycerol; 0.2 % (w/v) bromophenol blue
separating gel	10 % acryl amide; 375 mM Tris/HCl pH 8.8; 0.1 % (w/v) SDS; 0.5 % APS; 0.05 % TEMED
stacking gel:	4 % acryl amide; 125 mM Tris/HCl pH 6.8; 0.1 % (w/v) SDS; 0.5 % APS; 0.05 % TEMED
electrophoresis buffer:	25 mM Tris; 192 mM glycin; 0.1 % (w/v) SDS

2.2.12.3 Transfer of proteins and immunodetection

Protein patterns resolved by SDS-PAGE were transferred onto nitrocellulose membranes (Hybond-C extra, Amersham) by electrotransfer in a Mini TransBlot Cell system (BioRad). Following PAGE, gels were incubated in transfer buffer for 10 min. Whatman 3MM paper, pieces of fleece and the membrane were also pre-incubated in transfer buffer. The anode plate was overlaid with a layer of fleece, followed by two layers of 3MM paper, the membrane, the gel, two layers of 3MM paper and another layer of fleece. Electrotransfer was allowed to proceed at 100 V for 1h.

Membranes were subsequently blocked in TBS-T containing 5 % (w/v) skim milk powder for at least 1h. After that the membrane was incubated with the primary antibody diluted in skim milk powder (Table 5) for at least 1h, washed 2x 10 min in TBS-T, incubated with the secondary antibody diluted in TBS-T (Table 5) for at least 1h and washed 4x 10 min in TBS-T. Visualization of band signals was performed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce) according to manufacturer's instructions. X-ray films were developed and fixated according to standard photographic conditions.

TBS	10 mM Tris/HCl pH 7.5; 0.2 % (w/v) SDS
TBS-T	0.1 % (v/v) Tween 20 in TBS
transfer buffer	15.6 mM Tris; 120 mM glycin

Tab. 5: Antisera

antibody	properties	dilution	supplier
anti-atp1	raised against F_1-ATPase subunit 1 of maize	1:5000	A. Fernie, MPI Golm
anti-cox1	raised against cytochrome c-oxidase subunit 1 of maize	1:5000	A. Fernie, MPI Golm
anti-VDAC	raised against voltage-dependent anion selective channel protein of the outer membrane of potato mitochondria	1:1000	H.-P. Braun, Leibniz University of Hannover
secondary antibody	anti-rabbit IgG-horseraddish peroxidase conjugate	1:10,000	Sigma

3 Results

3.1 Expression analyses of phage-type RNA polymerase (*RpoT*) genes

3.1.1 Light-induced regulation of *RpoT* gene expression in *Arabidopsis thaliana* seedlings

It has been shown previously that expression of the three nuclear genes coding for organellar phage-type RNA polymerases, *RpoTp*, *RpoTm* and *RpoTmp*, is light induced in *Arabidopsis thaliana* seedlings (Preuten, 2004). In that study, steady-state transcript levels of the *RpoT* genes in 7-day-old dark-grown seedlings that were exposed to white light for up to twelve hours were analyzed. The results clearly showed a light-induced transcript accumulation of all three *RpoT* genes within six hours after illumination.

In the present study the experiments were repeated utilizing more sensitive assays and equipment. The results of the previous study were fully reproducible and confirmed a light-induced regulation of *RpoT* gene expression (Fig. 1). Light induction was most obvious for *RpoTp* (Fig. 1A) which codes for the plastid targeted polymerase, but also highly significant for both *RpoTm* (Fig. 1C) and *RpoTmp* (Fig. 1B) which encode the enzymes targeted to mitochondria and both organelles, respectively. Illumination of up to one hour did not cause any changes in the amount of transcripts of the three genes. After six hours, transcript levels of *RpoTmp* and *RpoTm* doubled compared to the dark control (samples taken after seven days in darkness without further illumination) and further increased more than threefold after twelve hours in light. *RpoTp* transcript levels were already increased roughly fivefold after six hours and further increased more than ninefold as compared to dark control levels.

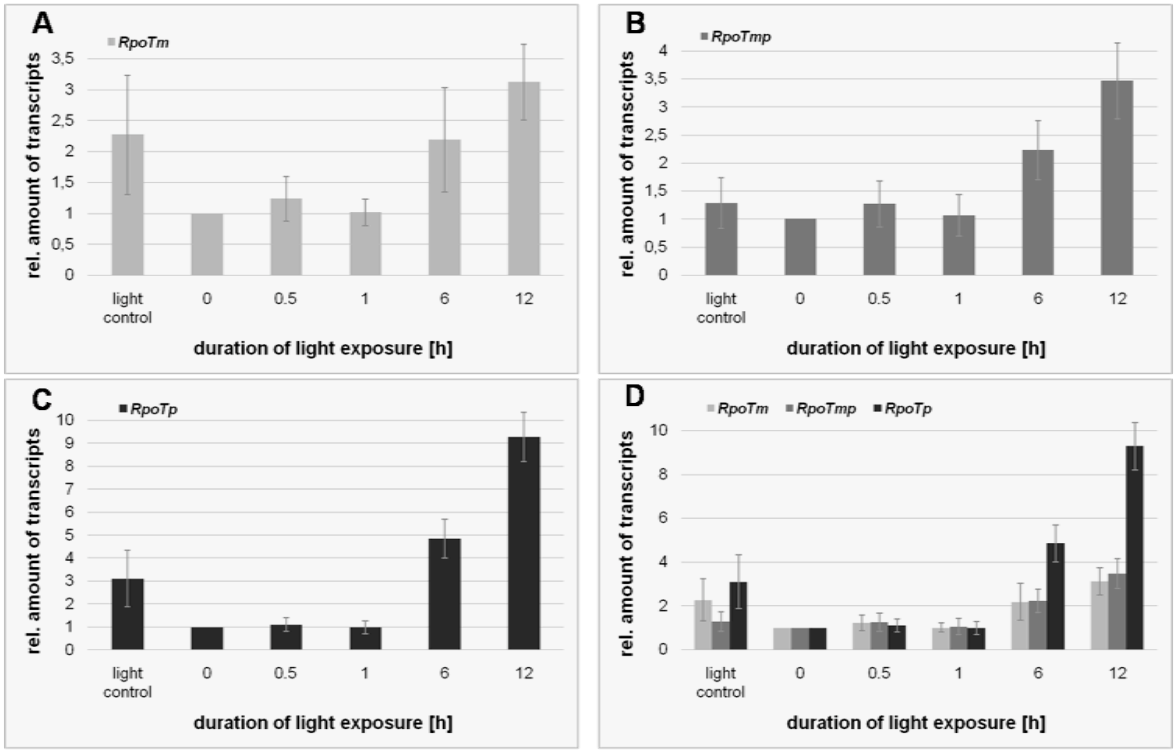


Figure 1: Accumulation of *RpoT* gene transcripts in *Arabidopsis* seedlings.
Wildtype (Col-0) seedlings were grown in darkness for 7 days and subsequently exposed to white light. Samples were taken in darkness (0) and after 30 minutes, one, six and twelve hours of illumination. An additional light control was grown under long-day conditions and harvested after 7 days. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

3.1.2 Light-induced regulation of *RpoT* gene expression in *Arabidopsis thaliana* rosette leaves

Etiolated seedlings of *Arabidopsis thaliana* show various typical morphological characteristics such as an elongated and unstable hypocotyl, a pronounced apical hook and undifferentiated chloroplast precursors leading to yellowish and small cotyledons (Symons *et al.*, 2008). De-etiolation or photomorphogenesis is a fundamental process in plant development and invokes several extensive changes not only in morphology, but also in biochemistry and gene expression (Dhingra *et al.*, 2006; Lin, 2002; Symons *et al.*, 2008).

These extreme conditions could as well lead to an altered expression of the *RpoT* genes that is not directly caused by light. To avoid the influence of the processes taking place during de-etiolation, light-induced regulation of the *RpoT* genes was subsequently analyzed in adult rosette leaves.

3.1.2.1 Light-induced expression of *RpoT* genes in adult rosette leaves

For these analyses, *Arabidopsis* plants were grown for three weeks under normal long-day conditions, darkened for three days and subsequently exposed to white light for up to twelve hours. The results showed highly increased steady-state levels of all analyzed transcripts after six hours and a decrease after twelve hours (Fig. 2). Again, effects were most pronounced for *RpoTp* transcripts (Fig. 2C) but also highly significant for *RpoTmp* and *RpoTm* mRNAs (Fig. 2B and A, respectively). After one hour, transcript levels did not show any changes. After six hours, *RpoTp* transcripts increased around 15-fold compared to dark controls. *RpoTm* and *RpoTmp* transcripts accumulated to four and seven times higher values, respectively. After twelve hours of illumination steady-state levels of *RpoTp* transcripts were decreased again by one third, *RpoTmp* and *RpoTm* transcript levels were reduced by two thirds and 50%, respectively.

These findings strongly suggest a light-induced regulation of *RpoT* gene expression in *Arabidopsis*, leading to accumulation of transcripts under white light. Hence, the previously found increase of transcript levels in etiolated seedlings exposed to light (see 3.1.1) is most probably also caused by light rather than by de-etiolation effects alone.

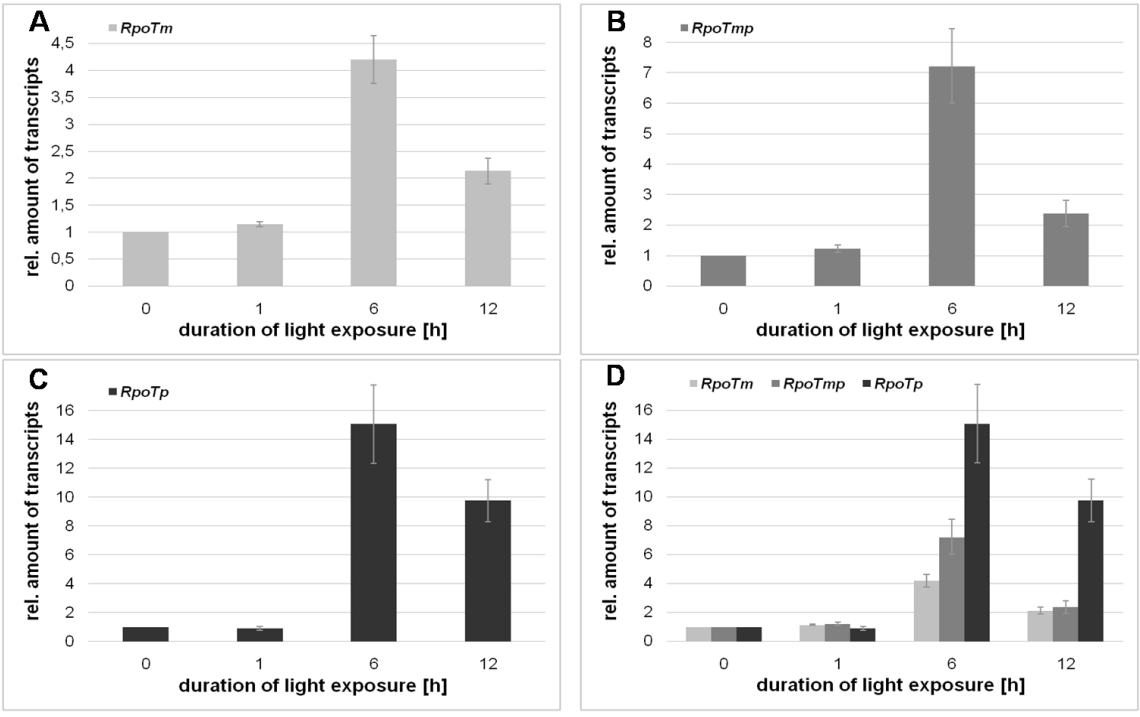


Figure 2: Accumulation of *RpoT* gene transcripts in rosette leaves of *Arabidopsis*.
Wildtype (Col-0) plants were grown under long-day conditions for 3 weeks, darkened for 72h and subsequently exposed to white light. Samples were taken in darkness (0) and after one, six and

twelve hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

3.1.2.2 Light-induced expression of *RpoT* genes in senescent rosette leaves

In the last few years there have been contradictory reports on the maintenance of plastid genomes during age-related senescence in *Arabidopsis* leaves (Li *et al.*, 2002; Rowan *et al.*, 2004; Zoschke *et al.*, 2007). Rowan *et al.* (2004) reported little or no plastid DNA being detectable by 4‘,6-diamidino-2-phenylindole (DAPI) staining in mature leaves even long before the onset of senescence. In contrast, results of Southern blot analyses using plastome-specific probes suggested that the amount of plastidial DNA in *Arabidopsis* leaves was relatively constant (Li *et al.*, 2006). The latter was supported by quantitative real-time PCR analyses. Zoschke *et al.* (2007) showed that plastome copy numbers varied from about 1000 to 1700 per cell without significant variation during development from young to old rosette leaves. However, transcription activity of plastid genes was significantly reduced in older rosette leaves.

Considering the existing data it was interesting to investigate the expression of *RpoT* genes, especially *RpoTp*, in senescent rosette leaves. To this end, *Arabidopsis* plants were grown in a long-day regime for nine weeks. After darkening for three days they were exposed to white light for up to twelve hours. Leaves showing signs of early age-related senescence (Fig. 3) were harvested and transcript levels of the *RpoT* genes were analyzed by quantitative real-time PCR.

Figure 2: Nine week old *Arabidopsis* plants.

Plants were grown in long-day conditions for nine weeks, darkened for 72h and subsequently exposed to light for up to twelve hours. Leaves showing signs of early senescence (violet color; marked in the photo) were chosen for the analyses.

Transcript levels showed a pattern very similar to what was observed in younger rosette leaves (see 3.1.2.1). After one hour of illumination no changes could be detected. In senescent leaves exposed to light for six hours steady-state levels were increased drastically. Again, the effect on *RpoTp* transcripts (Fig. 4A) was most striking with levels being increased more than elevenfold. Transcripts of *RpoTmp* (Fig. 4B) and *RpoTm* (Fig. 4C) accumulated to approximately 3.5 times higher levels compared to dark controls. As in 3-week old rosette leaves the amount of mRNAs was markedly reduced again after twelve hours in light. Altogether these results showed that even in senescent rosette leaves of *Arabidopsis RpoT* gene expression can be strongly induced by white light.

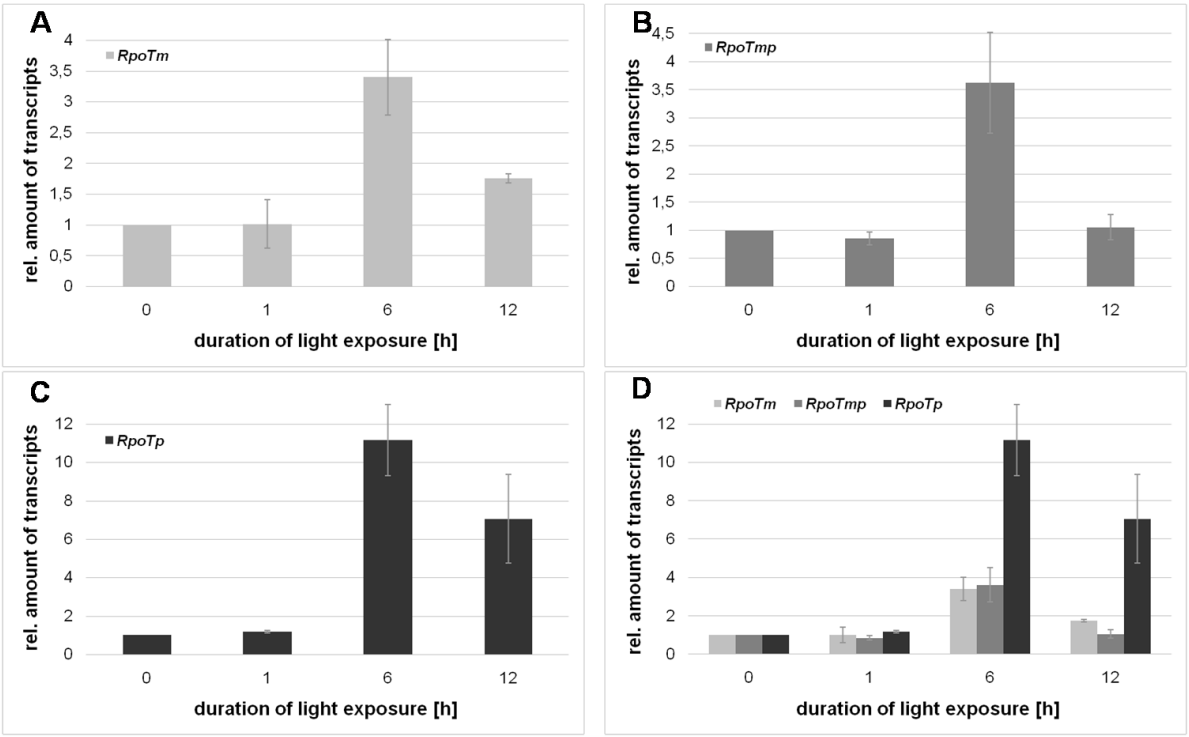


Figure 4: Accumulation of *RpoT* gene transcripts in senescent rosette leaves of *Arabidopsis*.
Plants were grown as described in Fig. 3. Samples were harvested in darkness (0) and after one, six and twelve hours in light. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

3.1.3 Circadian clock regulated expression of *RpoT* genes in *Arabidopsis*

Especially for plants as immotile organisms it is of fundamental importance to be able to react to changing environmental conditions, such as daily light/dark cycles. As this is a recurrent and predictable phenomenon most organisms, including plants, have developed diurnal rhythms to regulate multiple physiological and molecular processes. Some of these processes are direct responses to changing conditions, but some persist under constant conditions. Persistent rhythms that have periodicities matching that of the Earth's rotation on its axis (approximately 24h) are termed circadian. The mechanisms regulating these processes are called the circadian clock (Barak *et al.*, 2000; Gardner *et al.*, 2006).

Recent studies revealed roughly one third of the *Arabidopsis thaliana* transcriptome to be regulated by the circadian clock (Covington *et al.*, 2008; Schaffer *et al.*, 2001).

In order to determine its role in the expression of *RpoT* genes, circadian clock controlled accumulation of their transcripts was analyzed. For this purpose, plants were grown in 12h light/12h darkness cycles for three weeks. By then they had acquired a diurnal rhythm synchronized to the light/dark cycles. Subsequently the plants were illuminated constantly. After twenty-four hours in light transcript levels were measured for forty-eight hours. Any diurnal rhythm persisting under these conditions could then be attributed to a circadian regulation.

The experiments revealed a diurnal regulation of all *RpoT* gene transcript levels with similar peaks of accumulation in late night to early morning (Fig. 5A). Compared to transcript levels of *CCA1*, a key clock component gene (Fig. 5B), the variations found in *RpoT* transcript accumulation were relatively small, however. The expression of *CCA1* is known to be under strict circadian control with highest transcript accumulation shortly after dawn (Mizoguchi *et al.*, 2002; Wang und Tobin, 1998).

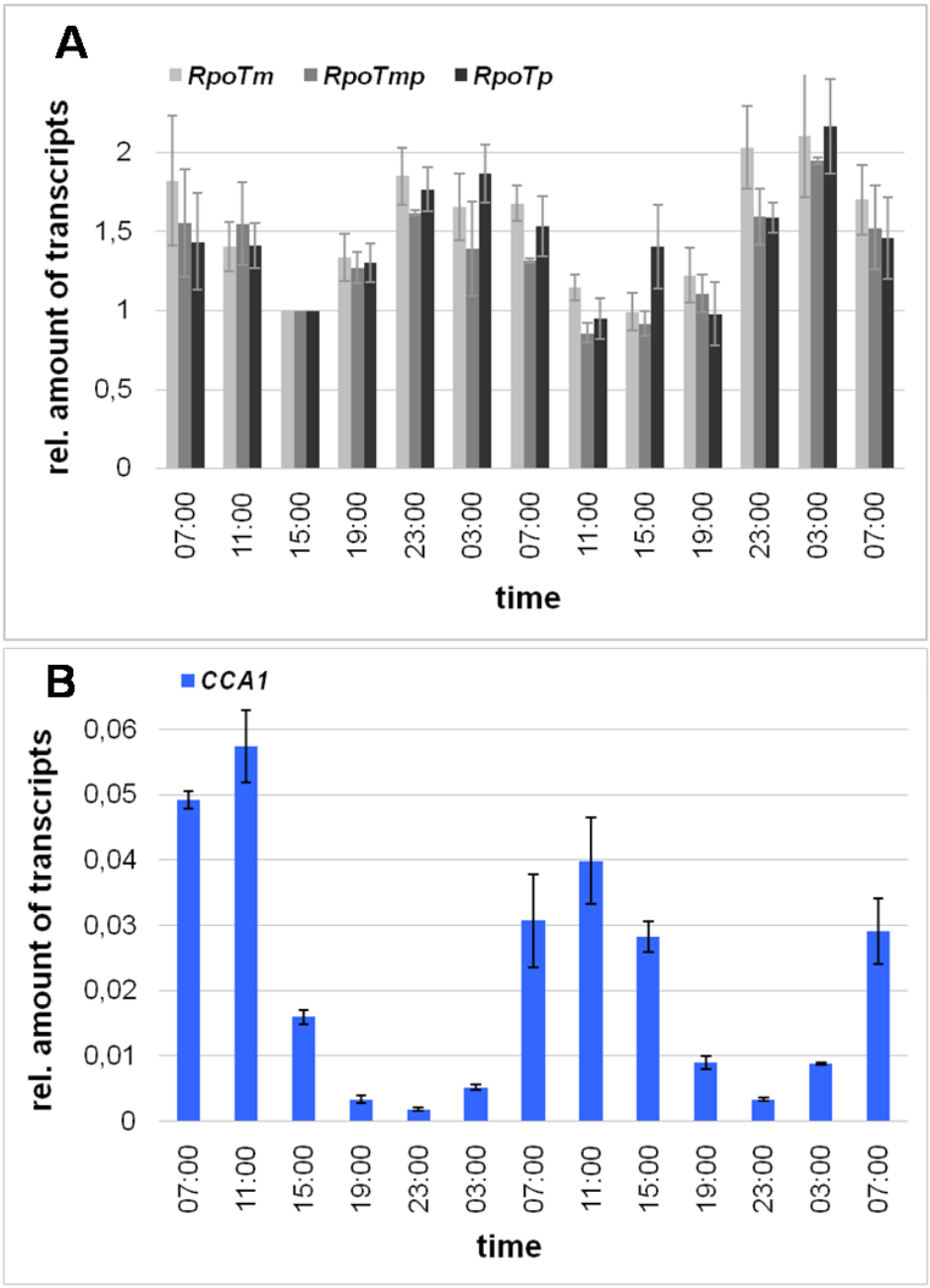


Figure 5: Circadian clock regulated accumulation of gene transcripts.
Arabidopsis plants were grown in a 12h light/12h dark regime for three weeks and subsequently exposed to constant light. After 24h harvesting of leaf samples began at 7:00 in the morning and was continued every four hours for a total of 48h. Relative amounts of *RpoT* gene (A) and *CCA1* (B) transcripts were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

The results thus showed that transcript accumulation of the *RpoT* genes is regulated by a circadian rhythm with, however, weak oscillation. These changes in the amount of transcripts cannot account for the strong induction found in illuminated seedlings and leafs (see 3.1.1 and 3.1.2), further suggesting a light induced expression of the three *RpoT* genes in *Arabidopsis thaliana*.

3.1.4 Analyses of *RpoT* gene expression in different light qualities

So far, transcript accumulation was analyzed only in white light. However, higher plants possess several different light receptors and are thus able to detect different light qualities (Chen *et al.*, 2004). The results described above revealed light induced regulation of *RpoT* gene expression. To learn more about the underlying pathways and photoreceptors playing a role in these processes, transcript accumulation was further analyzed in monochromatic red, blue and green light, achieved by different arrays of light emitting diodes (LED).

3.1.4.1 Transcript accumulation under red light

In higher plants, red/far-red light is sensed by the phytochrome family of photoreceptors (Salisbury *et al.*, 2007). The small gene family consists of five members, *PHYA-PHYE* (Clack *et al.*, 1994; Sharrock und Quail, 1989). *Via* phytochromes, red light triggers a lot of important processes such as seedling de-etiolation, vegetative architecture, apical dominance and the timing of reproductive development (Salisbury *et al.*, 2007). Absorption of red light promotes the conversion of the inactive P_r form of phytochromes into the active P_{fr} form, whilst far-red light reverses this process (Kendrick und Kronenberg, 1994; Schäfer *et al.*, 1972).

To get insight into the role of red light and phytochromes in the *RpoT* light induction pathway, 7-day-old etiolated *Arabidopsis* seedlings were exposed to monochromatic light with a wavelength of 631 nm. The results showed a different progression of transcript accumulation in red compared to white light (Fig. 6). While there was no change after one hour, *RpoTp* transcripts (Fig. 6C) were increased more than fourfold after four hours. Subsequently they declined continuously throughout further illumination. The amount of *RpoTmp* transcripts (Fig. 6B) also increased more quickly than in white light, being doubled after four hours. Transcripts accumulated even further after six hours and subsequently declined only marginally. Steady-state levels of *RpoTm* transcripts (Fig. 6A) were found to decline to about 50% after one hour. In the course of further illumination the values increased slowly, reaching a peak after six hours and subsequently decreased. Related to the dark control, mRNA levels were increased only 1.4 times in maximum.

Altogether, both *RpoTp* and *RpoTmp* transcript accumulation showed positive regulation by red light. *RpoTp* transcript levels increased most. Induction of transcript accumulation was faster but substantially weaker than in normal white light. *RpoTm* transcripts, however, showed a rather different progression. They decreased significantly quickly after

illumination and recovered after a few hours but showed almost no overall induction. Hence, substantial differences between the *RpoT* transcript accumulation patterns were observed in monochromatic red light.

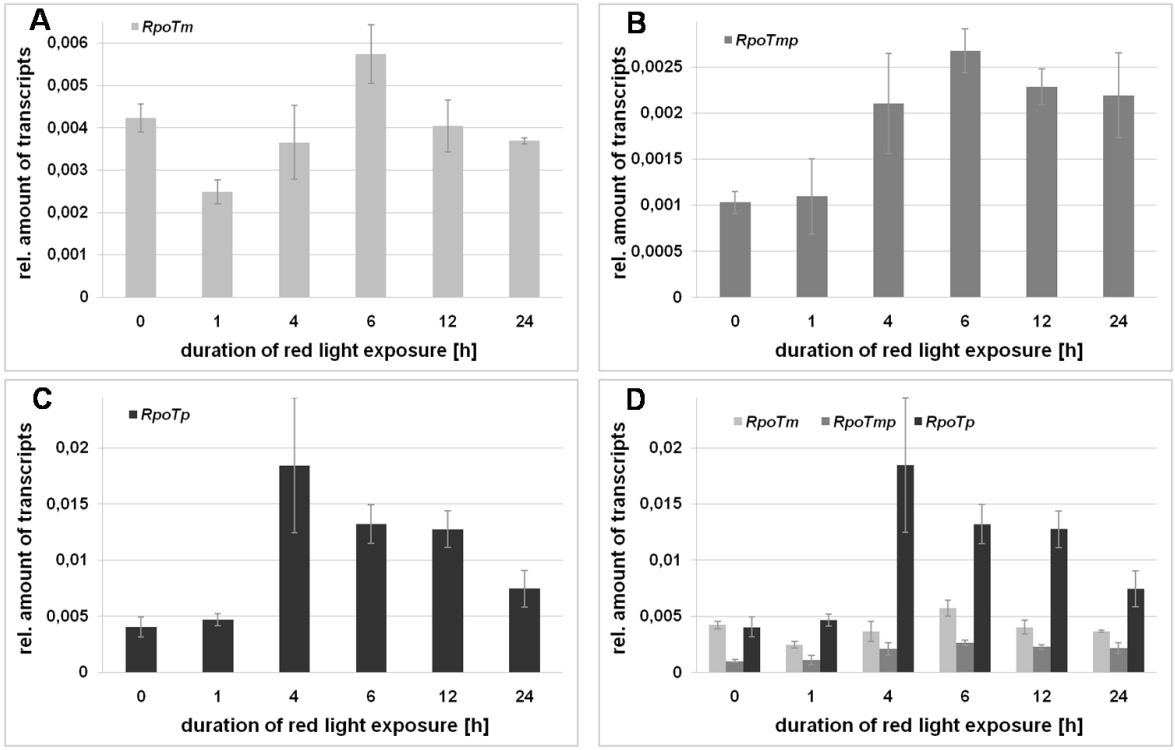


Figure 6: Accumulation of *RpoT* gene transcripts in red light.
Arabidopsis seedlings were grown in darkness for seven days and subsequently exposed to monochromatic light with a wavelength of 631 nm. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

3.1.4.2 Transcript accumulation under blue light

Higher plants possess two classes of UV-A/blue light receptors, phototropins and cryptochromes (Chen *et al.*, 2004; Lin, 2002). The cryptochrome gene family in *Arabidopsis* consists of two genes, *CRY1* and *CRY2* (Huang *et al.*, 2006; Toth *et al.*, 2001). Kleine *et al.* (2003) recently described a third cryptochrome gene termed *CRY-3* in *Arabidopsis thaliana*. Whether the gene product functions as a photoreceptor is yet unclear, though (Huang *et al.*, 2006; Kleine *et al.*, 2003; Selby und Sancar, 2006). Absorbing blue/UV-A light, cryptochromes mediate suppression of seedling stem elongation, promotion of cotyledon and leaf expansion, flowering time, resetting of the circadian oscillator and many other processes (Lin, 2002; Wu und Spalding, 2007).

To further investigate the role of blue light and cryptochromes in the processes leading to light induced expression of the *RpoT* genes in *Arabidopsis*, 7-day-old, etiolated seedlings were exposed to monochromatic light with a wavelength of 470 nm. The results revealed light induced accumulation of all *RpoT* gene transcripts with similar characteristics as observed in white light (see Fig. 1). However, overall transcript levels were slightly lower.

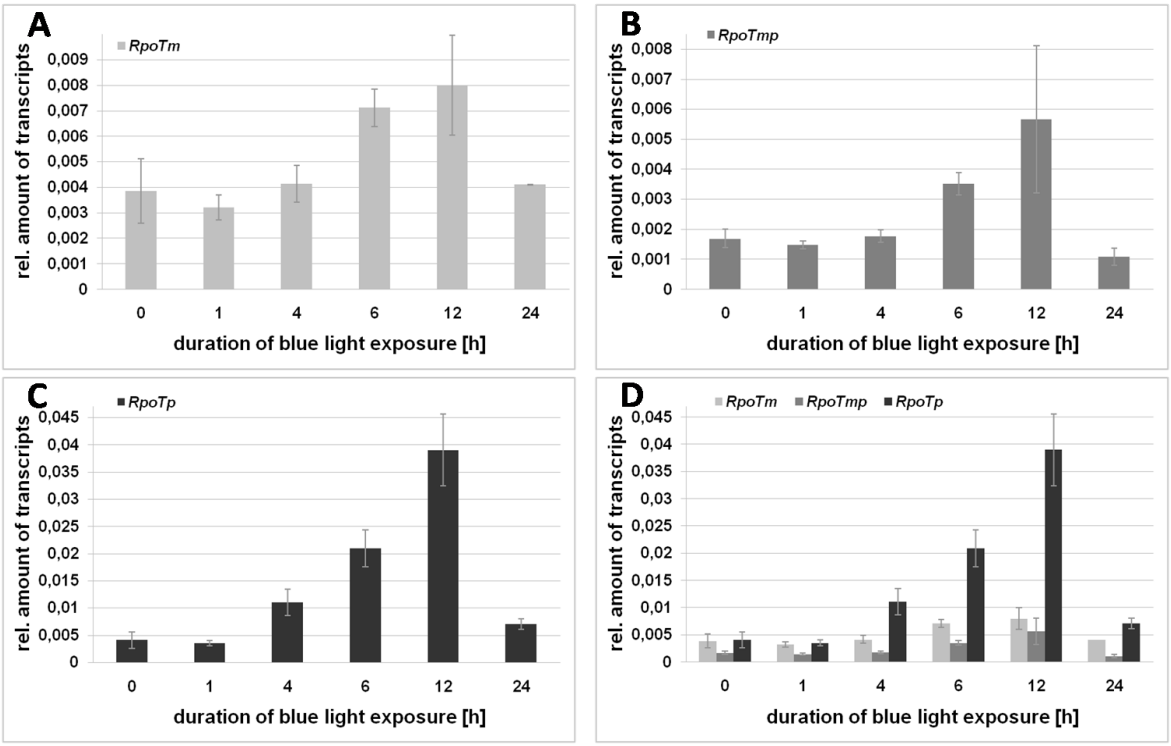


Figure 7: Accumulation of *RpoT* gene transcripts in blue light.
Arabidopsis seedlings were grown in darkness for seven days and subsequently exposed to monochromatic light with a wavelength of 470 nm. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

RpoTp transcript levels (Fig. 7C) did not show any changes after one hour, but doubled already after four hours. They further increased until at least twelve hours after illumination, reaching more than ninefold higher values compared to the dark control. After twenty-four hours in blue light the amount of transcripts was decreased drastically to almost the initial value. *RpoTmp* transcript levels (Fig. 7B) did not change until six hours of exposure. At that point, transcript abundance was doubled compared to dark controls. Transcript levels further increased to overall three times higher values after twelve hours, before dropping to less than initial levels in the twenty-four-hours-sample. Again, *RpoTm* (Fig. 7A) proved to be exceptional, showing only weak accumulation of transcripts after six hours (less than

twofold) and little further increase. Transcript levels were increased about twofold after twelve hours and decreased upon further illumination.

Overall, expression of all *RpoT* genes was found to be induced by blue light. Transcripts showed similar albeit weaker accumulation patterns as in broad-spectrum white light. The amount of *RpoTm* transcripts, however, only slightly increased after six hours and declined quickly afterwards.

3.1.4.3 Transcript accumulation under green light

Recently, the influence of green light on plant photomorphogenic development has been rediscovered. Folta (2004) could show that monochromatic green light causes an increase in seedling stem elongation, a response that is in opposition to that induced by all other light conditions studied (Folta, 2004). It had been shown previously that green light was able to inhibit seedling mass, plant cell culture growth and light induced gravitropic root elongation (Klein, 1992; Klein, 1964; Klein, 1979; Went, 1957). More recent studies showed that green light could reverse blue light-induced stomatal opening (Eisinger *et al.*, 2003; Frechilla *et al.*, 2000; Talbott *et al.*, 2006; Talbott *et al.*, 2003). Although phytochromes and cryptochromes readily absorb green light thereby influencing light induced events (Banerjee *et al.*, 2007; Bouly *et al.*, 2007; Lin *et al.*, 1995; Mandoli und Briggs, 1981; Shinomura *et al.*, 1996), there is supposed to be an additional, not yet discovered, distinct photosensory system for the absorption of green light (Dhingra *et al.*, 2006; Folta und Maruhnich, 2007).

In order to investigate the influence of green light on the *RpoT* gene light induction, 7-day-old etiolated *Arabidopsis* seedlings were exposed to monochromatic light with a wavelength of 525 nm.

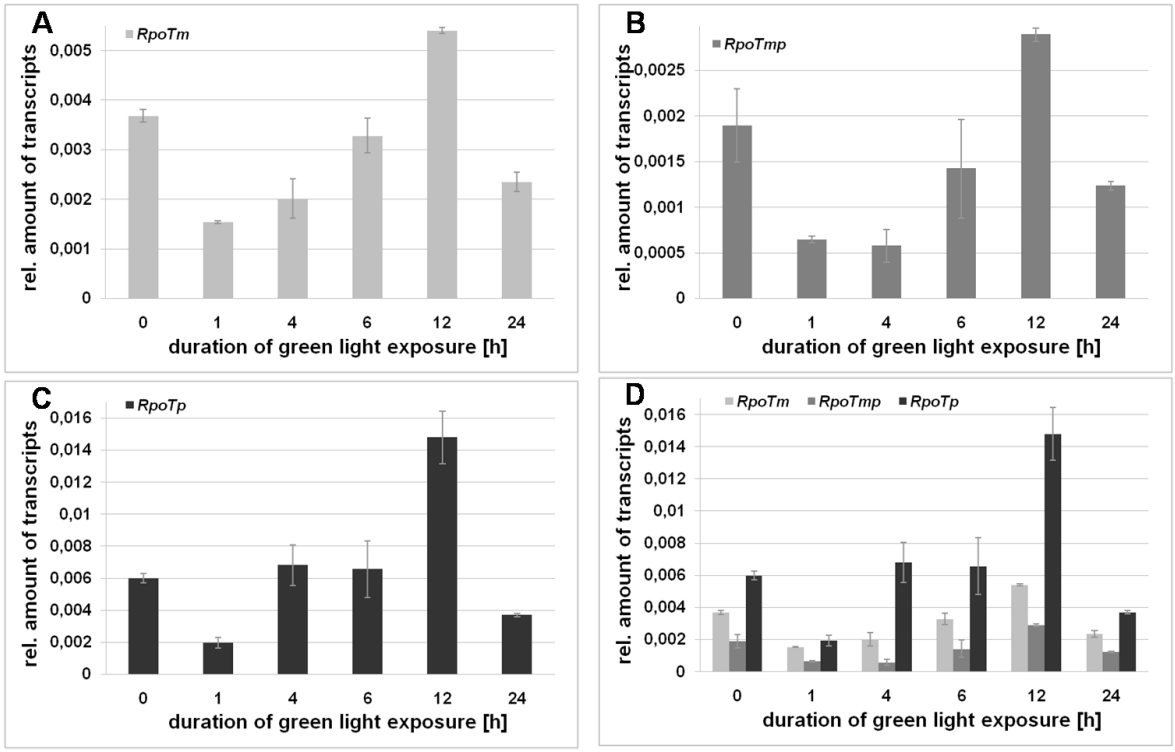


Figure 8: Accumulation of *RpoT* gene transcripts in green light.
Arabidopsis seedlings were grown in darkness for seven days and subsequently exposed to monochromatic light with a wavelength of 525 nm. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

All three genes showed a pronounced decrease of steady-state transcript levels after one hour of illumination (Fig. 8). The amount of transcripts was reduced 2.4-fold in the case of *RpoTm* (Fig. 8A) and even threefold in the case of *RpoTmp* (Fig. 8B) and *RpoTp* (Fig. 8C), respectively.

Three hours later *RpoTp* transcripts had recovered to initial levels, while no obvious change was observed for the other genes tested. *RpoTm* transcript levels reached the initial value after six hours and increased further until twelve hours in light. At this point of time an overall 1.5-fold increase was detected as compared to the dark control. Steady-state levels of *RpoTmp* showed a very similar progression. The net increase of *RpoTp* transcripts after twelve hours was, however, bigger. The amount of mRNA at this time point was 2.5 times higher than in the dark. Further illumination of the seedlings led to a drastic reduction of transcripts after twenty-four hours for all genes. Steady-state levels dropped notedly beyond the initial values.

Exposition to monochromatic green light obviously led to a transient negative regulation of *RpoT* gene expression in etiolated *Arabidopsis* seedlings. This reaction was much faster than any positive light induction observed for these genes so far. After only one hour transcript levels were found to be decreased up to three times. Further illumination of the seedlings led to an increase of transcripts back to levels found in darkness. However, net induction was very small. After one day of exposure, the amount of mRNAs was strongly decreased again. Hence, green light has a pronounced and unique effect on the expression of *RpoT* genes in *Arabidopsis* seedlings.

3.1.5 Analyses of *RpoT* gene expression in different photoreceptor mutants

Plants are dependent on photosynthesis for survival. As sessile organisms, however, they are not able to move when light conditions change or get worse. Therefore, they possess a battery of photoreceptors for sensing different wavelengths of the spectrum (Chen *et al.*, 2004). Knockout mutants of these photoreceptors are a useful tool to further investigate the pathways leading to light induction of distinct genes. For the experiments described in the following chapters mutants lacking one or several photoreceptor genes were grown in the same conditions as wildtype plants before (see 2.2.1) and transcript levels were analyzed by quantitative real-time PCR.

3.1.5.1 Expression of *RpoT* genes in different cryptochrome knockout mutants

Cryptochromes are UV-A/blue light absorbing photoreceptors which seem to be ubiquitous in the plant kingdom (Cashmore *et al.*, 1999). They were most probably evolutionary derived by gene duplication from DNA repair enzymes called photolyases. Although they share similarities in amino acid sequence and chromophore composition with photolyase, cryptochromes have not retained DNA repair activity (Cashmore *et al.*, 1999; Lin *et al.*, 1995). In *Arabidopsis* there are two proteins of the cryptochrome family, cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2). They play an important role in the de-etiolation of dark-grown seedlings and affect multiple processes including hypocotyl elongation, cotyledon opening, gene expression and chloroplast development. Additionally, they are involved in acquirement of circadian rhythms and photoperiodic flowering (Ahmad und Cashmore, 1993; Guo *et al.*, 1998; Lin, 2002).

3.1.5.1.1 *RpoT* transcript accumulation in *cry1* knockout mutants

The *CRY1* gene of *Arabidopsis* was first described and isolated in 1993 in a study of the *hy4* mutant (Ahmad und Cashmore, 1993). Mutant plants lacking CRY1 (referred to as HY4 previously) show decreased sensitivity to blue light, resulting in impaired inhibition of hypocotyl elongation in seedlings (Ahmad und Cashmore, 1993; Koornneef *et al.*, 1980).

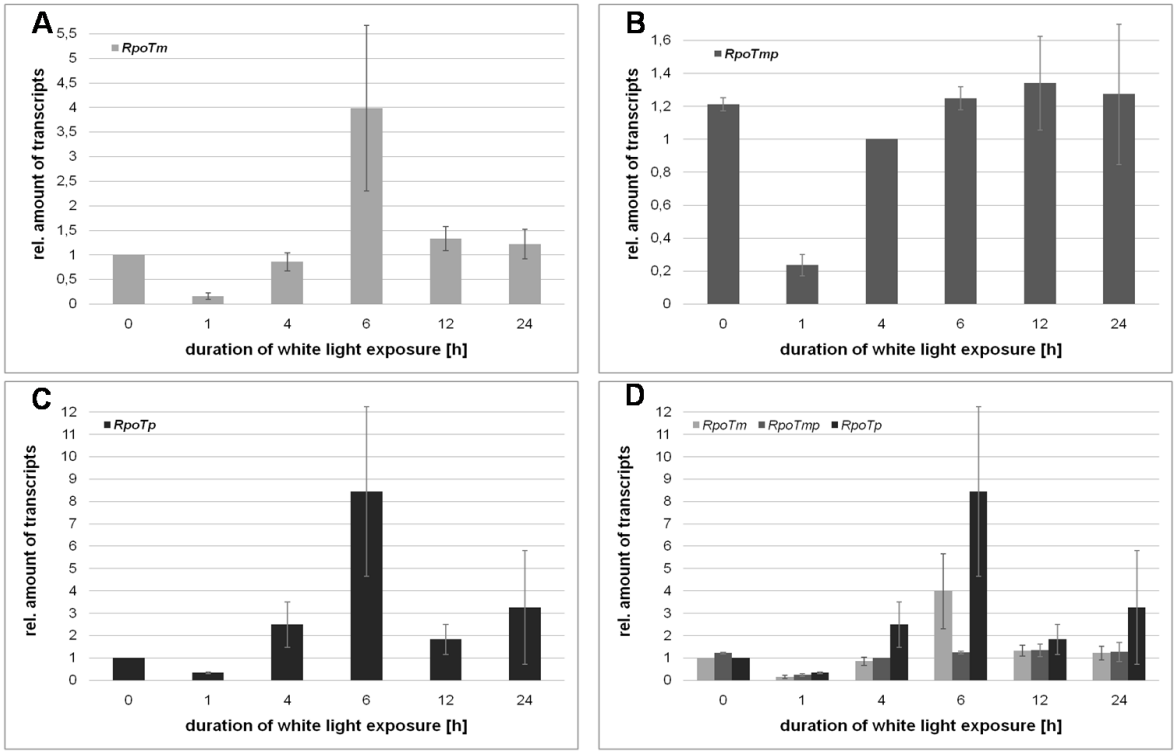


Figure 9: Accumulation of *RpoT* gene transcripts in *cry1* mutants.
Seedlings of *cry1* mutants were grown in darkness for seven days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

In order to investigate if the absence of CRY1 has any impact on the expression of *RpoT* genes, 7-day-old etiolated *cry1* mutant seedlings were exposed to white light for up to twenty-four hours. As shown in Figure 10, transcript levels of all *RpoT* genes displayed a strong decline quickly after exposition to light. *RpoTp* transcripts (Fig. 9C) were decreased threefold, *RpoTmp* transcripts (Fig. 9B) fivefold and those of *RpoTm* (Fig. 9A) even more than sixfold. After four hours of illumination the amount of *RpoTm* and *RpoTmp* mRNAs increased back to dark control values, whereas *RpoTp* transcript levels already exceeded the initial values. In case of *RpoTmp* further illumination did not invoke any significant changes, whereas *RpoTm* and *RpoTp* transcript levels were increased up to four- and

eightfold, respectively, after six hours. In the course of the experiment these levels decreased again. After twelve hours in light they had dropped to roughly initial amounts without any further changes.

Seedlings of *cry1* mutants obviously retained the ability to regulate *RpoTm* and *RpoTp* gene expression in a light dependent manner. This reaction, however, was preceded by a rapid and strong decrease of steady-state levels which has not been observed in the wildtype. Transcript accumulation of *RpoTmp* seemed to be affected most dramatically in these mutants, as it did not show any net increase.

3.1.5.1.2 *RpoT* transcript accumulation in *cry2* knockout mutants

The gene coding for the second cryptochrome in *Arabidopsis thaliana* was identified in 1996 and designated *CRY2* (Lin *et al.*, 1996). The CRY2 protein shows 51% identity to CRY1 in amino acid sequence. *cry2* mutants also show a long-hypocotyl phenotype, suggesting a role of CRY2 in the de-etiolation process similar to that of CRY1 (Guo *et al.*, 1998; Lin *et al.*, 1998). Hence, there is a functional redundancy between CRY1 and CRY2 (Mazzella *et al.*, 2001; Mockler *et al.*, 1999). However, other than *CRY1*, *CRY2* expression is negatively regulated by blue light and therefore it functions mainly under low light (Lin *et al.*, 1998). Additionally, there seems to be a complex functional interaction between cryptochromes and phytochromes in photomorphogenesis (Más *et al.*, 2000; Neff und Chory, 1998). Transcript levels of *RpoT* genes were analyzed in *cry2* mutant seedlings in order to elucidate the role of CRY2 in the light induction process.

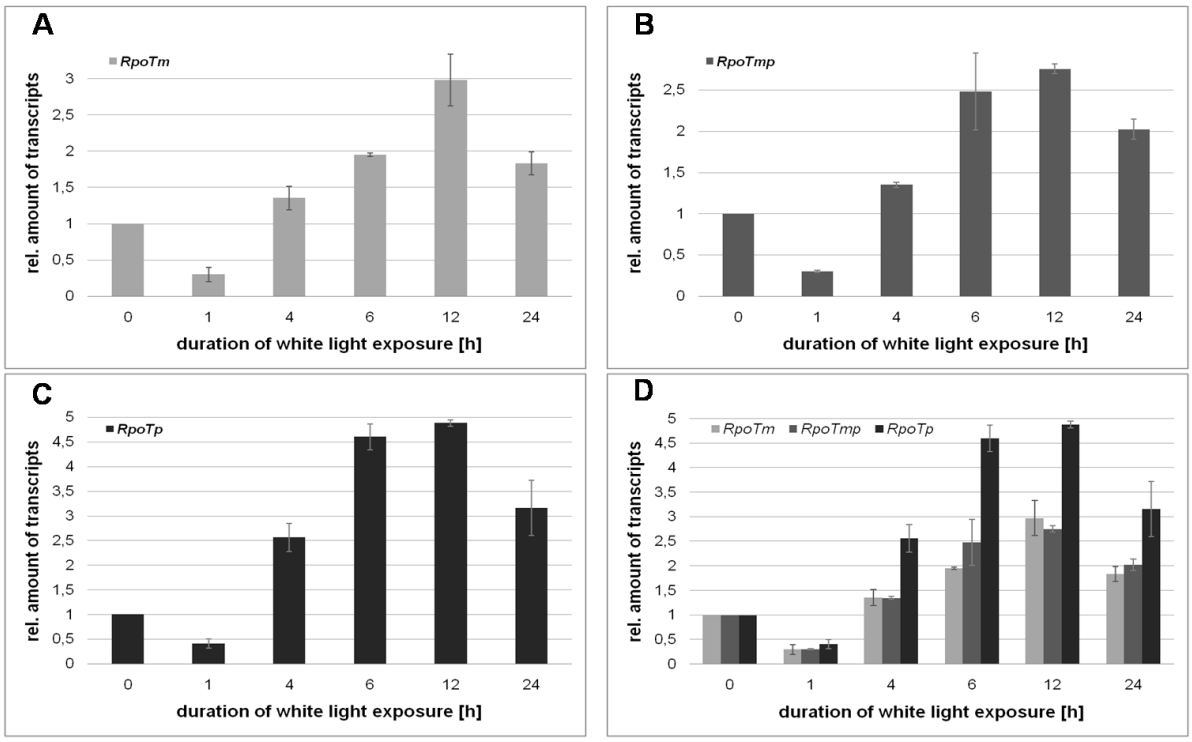


Figure 10: Accumulation of *RpoT* gene transcripts in *cry2* mutants.
Seedlings of *cry2* mutants were grown in darkness for seven days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

Similarly to the situation in *cry1* mutants (see 3.1.5.1.1), transcript abundance in *cry2* mutants declined rapidly after exposure to light (Fig. 10). However, the decrease was not as pronounced as in *cry1* mutants (2.5-fold for *RpoTp*, roughly threefold for *RpoTm* and *RpoTmp*). Upon further illumination the seedlings showed steadily increasing *RpoT* gene transcript accumulation until at least twelve hours after transfer to light. *RpoTp* transcripts (Fig. 10C) increased most, accumulating to almost five times higher levels after twelve hours in light compared to dark controls. The amount of *RpoTm* mRNA (Fig. 10A) after twelve hours was about three times higher than in darkened seedlings, and *RpoTmp* transcript levels (Fig. 10B) were increased slightly more than 2.5-fold. After exposure for twenty-four hours the amount of all transcripts was readily decreased again.

Apparently, *RpoT* gene expression in *cry2* mutants was light induced. Overall effects were less pronounced than in the wildtype. However, the characteristics of transcript accumulation were very similar, except for the rapid decrease shortly after exposure to light, which had also been found earlier in mutants lacking CRY1 (Fig. 9), but not in the wildtype.

3.1.5.1.3 *RpoT* transcript accumulation in *cry1/cry2* double knockout mutants

The *cry1*/*cry2* double knockout mutant has more severe phenotypic defects in photomorphogenesis than has either the *cry1* or *cry2* mutant (Mazzella *et al.*, 2001; Mockler *et al.*, 1999). This is apparently due to a functional redundancy of CRY1 and CRY2. Similar analyses as for the monogenic mutants were carried out for the double mutant in order to clarify how the total lack of cryptochromes affects light-driven *RpoT* gene expression.

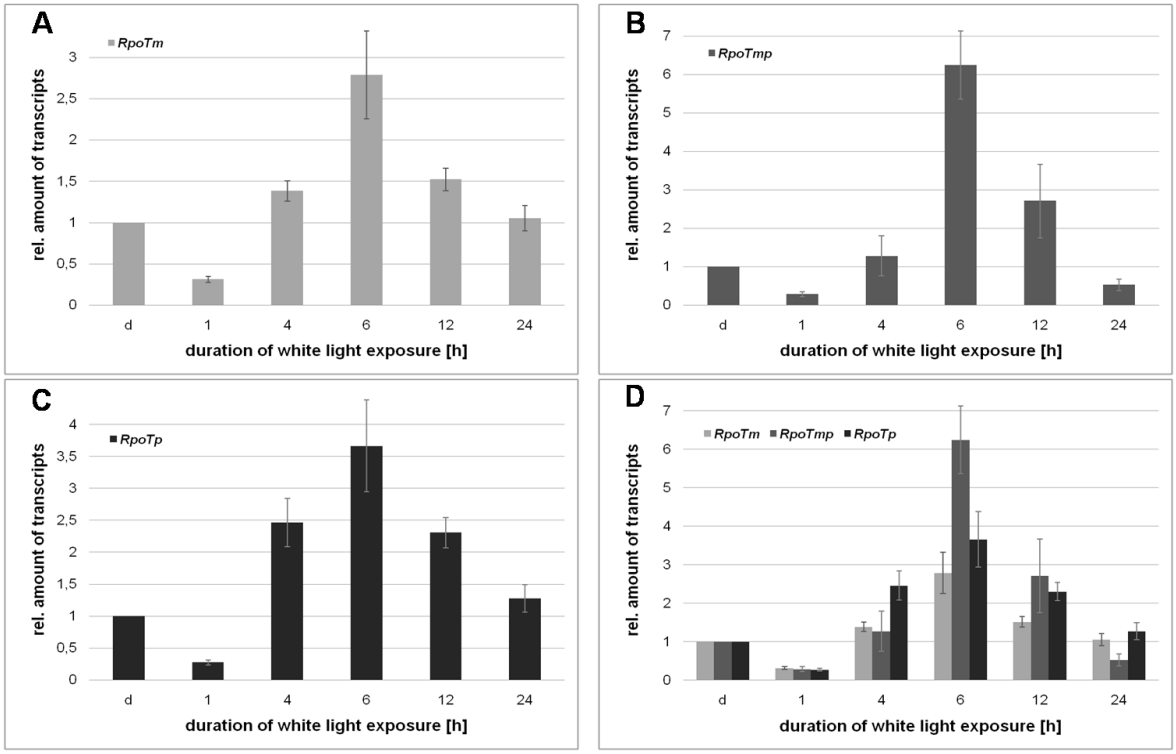


Figure 11: Accumulation of *RpoT* gene transcripts in *cry1/cry2* double mutants.
Seedlings of *cry1/cry2* mutants were grown in darkness for seven days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

Transcript levels of all three *RpoT* genes showed a similar characteristic course of light induction. Shortly after exposure, transcript accumulation dropped distinctly to less than one third of initial levels (Fig. 11). In the progress the amount of all *RpoT* transcripts increased continuously until reaching a peak after six hours of illumination. At that point transcript levels of *RpoTm* (Fig. 11A) were increased more than 2.5-fold compared to dark controls, those of *RpoTp* (Fig. 11C) more than 3.5-fold and those of *RpoTmp* (Fig. 11B) even more than sixfold. Upon further illumination transcript abundance decreased steadily. Values after

twenty-four hours approximated initial transcript levels of *RpoTm* and *RpoTp*. In the case of *RpoTmp* they even dropped to only half of the levels found in dark controls.

Even the loss of both cryptochrome genes did not inhibit the ability of the plant to regulate *RpoT* gene expression in a light induced fashion. Again, a rapid decline of transcript levels could be observed which obviously is characteristic of cryptochrome mutants, as it was also present in both single mutants (see Figs. 10 and 11). Interestingly, accumulation of *RpoTmp* mRNAs was induced most effectively in the double mutant, whereas it had not been stimulated at all in the *cry1* mutant (Fig. 9) and only weak in the *cry2* mutant (Fig.11).

3.1.5.2 Expression of *RpoT* genes in different phytochrome knockout mutants

Plants perceive red/far-red light signals using specialized photoreceptors, the phytochromes. In all higher plants analyzed so far, phytochromes are encoded by a small multigene family (Mathews und Sharrock, 1997). *Arabidopsis thaliana* possesses five genes of this family, *PHYA* through *PHYE* (Clack *et al.*, 1994; Sharrock und Quail, 1989). Unique among photoreceptors, phytochromes exist as two photoconvertible isomers, the P_r and P_{fr} form. They are synthesized in the inactive red light absorbing P_r form and acquire activity through photoconversion upon absorption of red light. The active far-red light absorbing P_{fr} form can be converted back by absorbing far-red light (Kendrick und Kronenberg, 1994). Multiple important processes in higher plants are controlled by phytochromes, including seed germination and seedling establishment, transition from vegetative to reproductive growth and shade avoidance (Chen *et al.*, 2004; Johnson *et al.*, 1994; Nagatani *et al.*, 1993; Reed *et al.*, 1994; Shinomura *et al.*, 1994; Whitelam *et al.*, 1993).

3.1.5.2.1 *RpoT* transcript accumulation in *phyA* knockout mutants

The *PHYA* gene encodes the apoprotein of phytochrome A (PHYA). This photoreceptor mainly mediates responses to very low radiance red and far-red light and dominates by far in etiolated seedlings. Upon exposure to light, PHYA is rapidly degraded and *PHYA* transcript abundance is reduced (Kendrick und Kronenberg, 1994). Null mutants of PHYA were simultaneously identified by several groups in screens for a long hypocotyl phenotype (Nagatani *et al.*, 1993; Parks und Quail, 1993; Whitelam *et al.*, 1993). They are completely insensitive to far-red light (Barnes *et al.*, 1996; Johnson *et al.*, 1994; Whitelam *et al.*, 1993). As other phytochrome mutants do not show this phenotype, PHYA is considered to be the

sole photoreceptor promoting seedling responses to this wavelength, whereas its role in red light seems to be only minor (Boylan und Quail, 1991; Quail *et al.*, 1995).

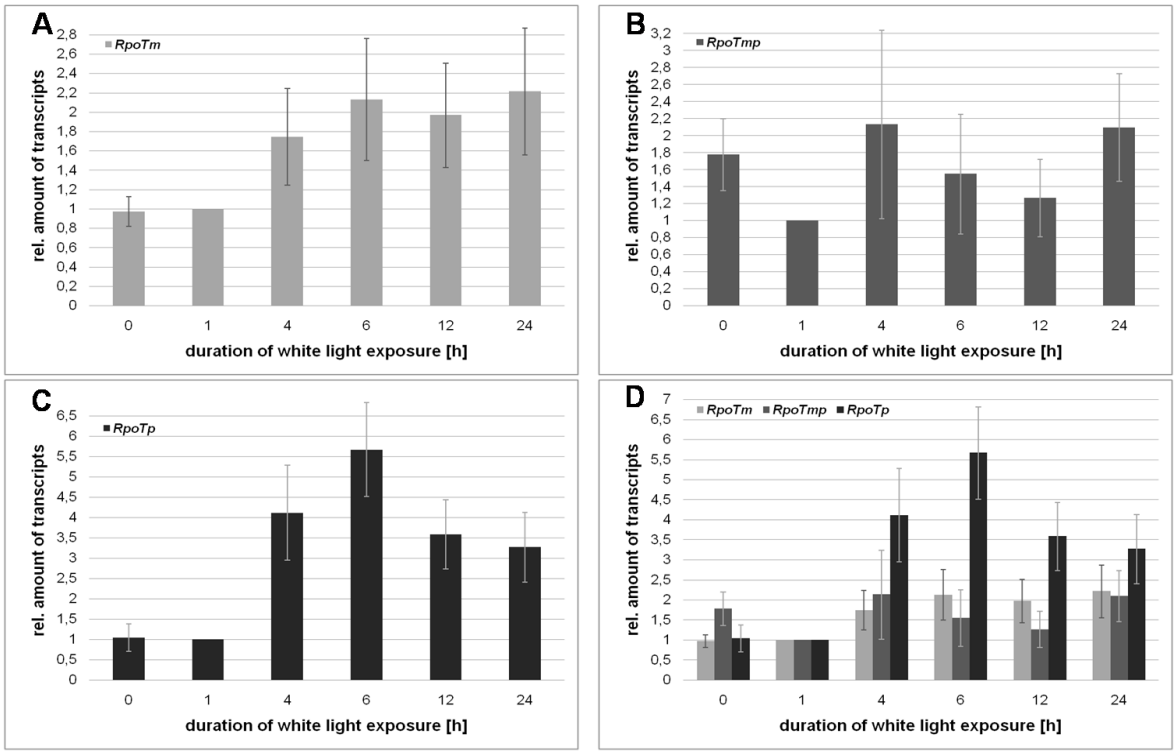


Figure 12: Accumulation of *RpoT* gene transcripts in *phyA* mutants.
Seedlings of *phyA* mutants were grown in darkness for seven days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

In order to get a deeper insight into the function of phytochrome A during the light induction process of *RpoT* genes in *Arabidopsis*, *phyA* mutants were grown in darkness for seven days and subsequently exposed to white light for up to twenty-four hours. Obviously, expression of the *RpoT* genes was affected differentially by the loss of PHYA. *RpoTp* showed clearly light induced accumulation of its transcripts (Fig. 12C). While there was no change after one hour in light, transcript levels were increased fourfold after four hours and almost 5.5-fold after six hours. Upon further exposure to light the amount of transcripts decreased, but remained higher than in dark controls even after twenty-four hours. *RpoTm* transcripts (Fig. 12A) showed much weaker effects. As in the case of *RpoTp* there was no alteration after one hour in white light. After four hours mRNA levels were slightly increased and did not change significantly further. The accumulation pattern of *RpoTmp* transcripts (Fig. 12B) was affected considerably in *phyA* mutants. Exposure to light did no

longer have any positive effect on the amount of mRNA. In contrast, a decline of transcripts after one hour was observed. After four hours transcript levels recovered to initial values and subsequently slightly decreased again. After one day of illumination a roughly 1.5-fold increase was found. Overall, however, illumination did not induce an increase of *RpoTmp* transcript levels.

Thus, the lack of phytochrome A had distinct effects on light induced expression of the *RpoT* genes in *Arabidopsis*. Whereas *RpoTp* transcript accumulation was clearly induced by exposure to white light for several hours, *RpoTmp* mRNA did no longer show positive regulation. *RpoTm* transcripts increased slightly after some hours without being regulated further.

3.1.5.2.2 *RpoT* transcript accumulation in *phyB* knockout mutants

The *PHYB* gene of *Arabidopsis thaliana* encodes the photoreceptor PHYB, a light stable phytochrome. It is less abundant than PHYA and is not degraded upon irradiation (Kendrick und Kronenberg, 1994). Mutants deficient in PHYB were first described in 1980. At that time they were called *hy3*, for the long hypocotyl phenotype by which they were recognized (Koornneef *et al.*, 1980; Somers *et al.*, 1991). These mutants show reduced inhibition of hypocotyl elongation, accumulate less chlorophyll and flower earlier than the wildtype (Koornneef *et al.*, 1980; Reed *et al.*, 1993). They also show an altered shade avoidance response (Smith und Whitelam, 1997). However, *phyB* mutants de-etiolate normally when grown under far-red or blue light (Koornneef *et al.*, 1980; Reed *et al.*, 1993). Taken together, this indicates that, while PHYA controls photomorphogenesis under far-red light, PHYB controls the same processes under red light.

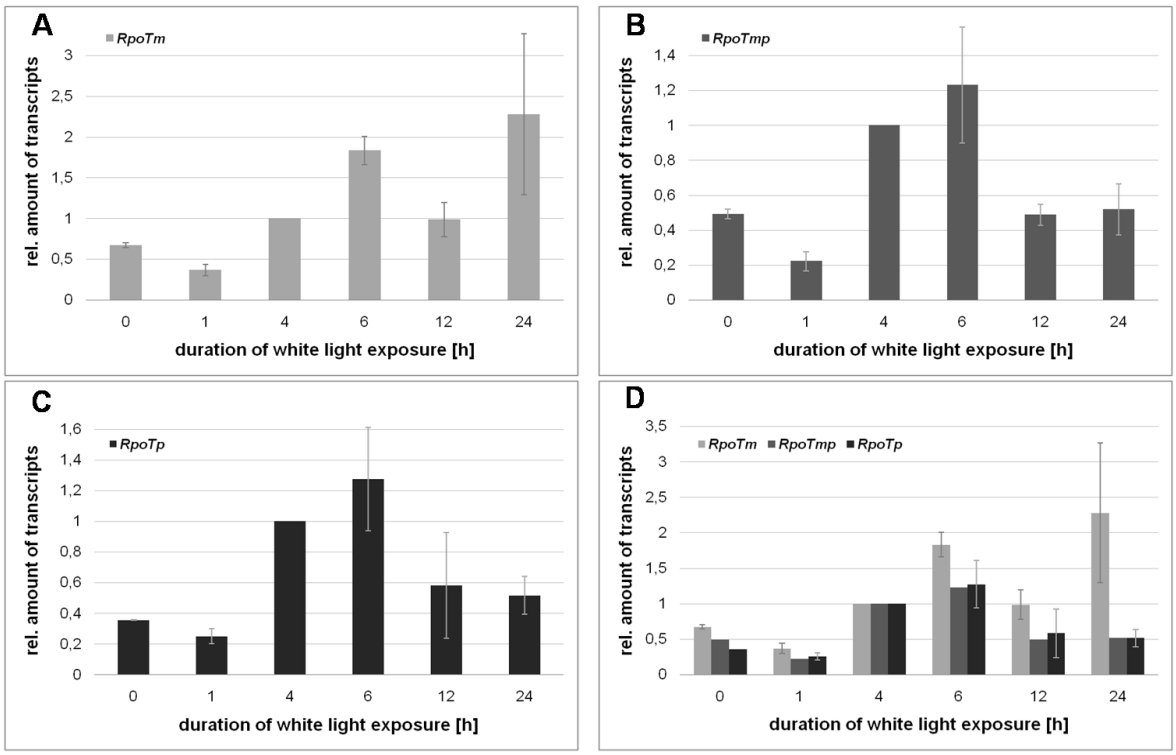


Figure 13: Accumulation of *RpoT* gene transcripts in *phyB* mutants.
Seedlings of *phyB* mutants were grown in darkness for seven days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

Knockout of the *PHYB* gene leading to plants lacking phytochrome B had less severe effects on light induced expression of *RpoT* genes than has the knockout of *PHYA*. *RpoTp* transcript accumulation, however, proceeded very similar in both single mutants. As in the *phyA* mutant, transcripts levels increased, albeit not as much as in the former, until reaching a maximum after six hours of illumination (Fig. 13A). At that point they were increased 3.5-fold. Upon further illumination levels decreased again and remained slightly higher than in dark controls. Unlike in the *phyA* mutant, a slight decline in the amount of mRNA could be observed after one hour in light in the *phyB* mutant. The same effect was also observed for *RpoTm* and *RpoTmp* transcript levels (Fig. 13A and B, respectively). Similar to *RpoTp* transcripts, the mRNA abundance of the other *RpoT* genes first declined quickly and afterwards increased until reaching a maximum after six hours of illumination. Both increases were in the same range, with peak values being approximately 2.5 times higher than those in dark controls. After twelve hours steady-state levels of both *RpoTm* and *RpoTmp* transcripts had dropped significantly. In case of *RpoTmp* they reached approximately the initial values of dark controls and stayed constant upon further

illumination. Abundance of *RpoTm* transcripts did not decline as much and additionally tended to increase again after twenty-four hours.

Overall, lack of phytochrome B did not lead to comparably severe effects on the expression of *RpoTmp* and *RpoTm*, as did the loss of PHYA. Transcript accumulation of all three *RpoT* genes was still found to be regulated in a light induced manner. Interestingly, after a short period of illumination transcript levels of all three genes temporarily declined. A similar effect has also been observed in different cryptochrome mutants (see 3.1.5.1).

3.1.5.2.3 *RpoT* transcript accumulation in *phyA/phyB* double knockout mutants

Evolution of distinct photosensory roles for different phytochromes has been allowed by diversification of the *PHY* gene family. While PHYA and PHYB have divergent functions, the more closely related PHYC, PHYD and PHYE possess overlapping or even redundant roles (Mathews und Sharrock, 1997). Knocking out both *PHYA* and *PHYB* depletes the two most important phytochromes and severely effects germination and seedling development in red and far-red rich light. The effect of a loss of both PHYA and PHYB on light induced *RpoT* gene expression was studied using a *phyA/phyB* double mutant. Seedlings were grown and analyzed under the conditions described above

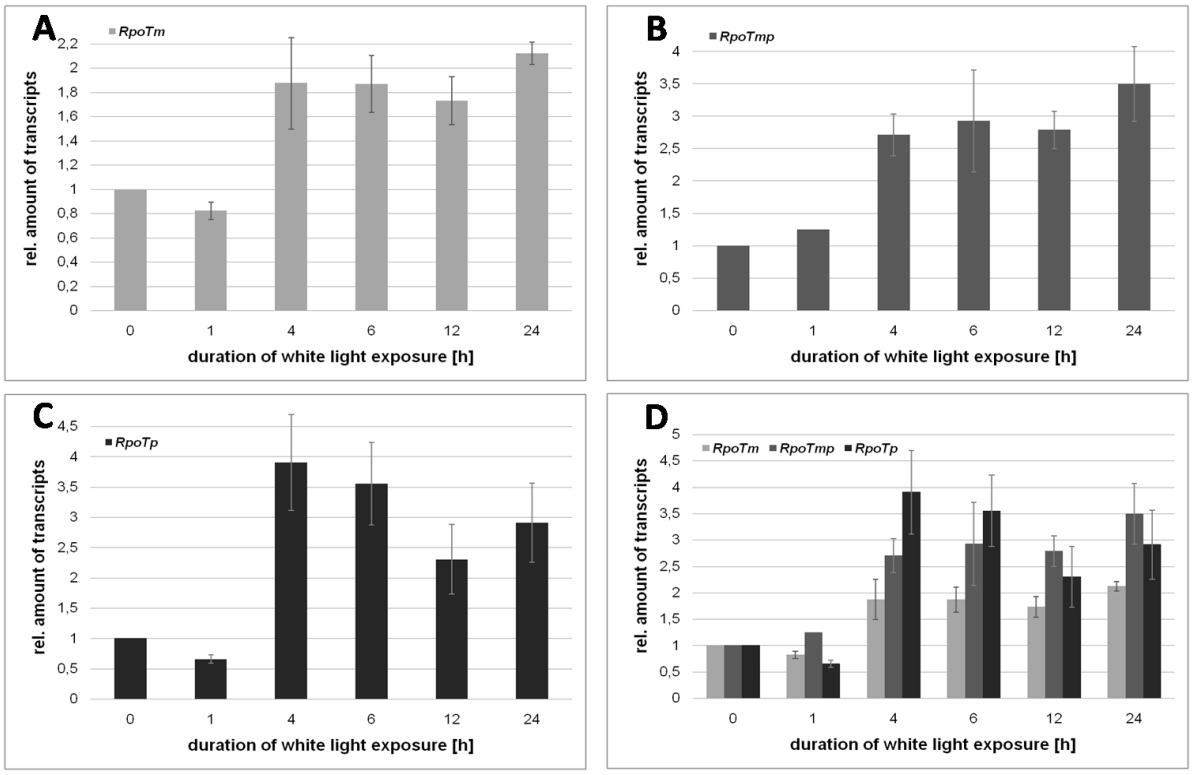


Figure 14: Accumulation of *RpoT* gene transcripts in *phyA/phyB* double mutants.
Seedlings of *phyA/phyB* mutants were grown in darkness for seven days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

As shown in Figure 15, lack of both main phytochromes, PHYA and PHYB, had articulate effects on *RpoT* gene expression. Accumulation of *RpoTm* transcripts proceeded very similar to what has been observed in *phyA* single knockout mutants. Transcript levels increased between 1.5- and twofold upon illumination and did not change during the progression of the experiment (Fig. 14A). In both cases, transcript abundance did not change before four hours of illumination. *RpoTmp* transcript levels behaved similarly, yet showing a bigger overall increase (Fig. 14B). In average the amount of mRNA in light was 2.5- to 3.5 times higher than in darkness. As seen for *RpoTm* transcript levels, an increasing tendency was not detectable before four hours of illumination. Again, accumulation of *RpoTp* transcripts was found to be most distinctly induced by light (Fig. 14C). Interestingly, transcript levels were slightly decreased after one hour, but increased strongly upon further illumination. After four hours the amount of mRNAs was around fourfold higher than in dark controls. Subsequently, however, levels did not increase further, but declined roughly 1.5 times until twelve hours of light exposure, not changing significantly afterwards.

Knockout of the two most important phytochrome genes, *PHYA* and *PHYB*, leads to broadly changed transcript accumulation patterns of all *RpoT* genes. *RpoTm* transcript levels showed a rather small increase and did not change further upon illumination. Levels of *RpoTmp* transcripts were increased stronger but also did not show any further regulation. Only *RpoTp* mRNA levels still showed a more distinct pattern of light regulation, being notably increased after four hours and decreasing again upon further illumination. However, progression broadly differed from that in the wildtype.

3.1.5.3 Expression of *RpoT* genes in *hy5* knockout mutant

The basic domain/leucine zipper (bZIP) protein HY5 was the first known and is the most extensively studied transcription factor involved in promoting photomorphogenesis. It was first identified in a screen for mutants with a long hypocotyl phenotype (Koornneef *et al.*, 1980). The *hy5* mutants showed a defect in inhibition of hypocotyl elongation under all light conditions, suggesting a function of HY5 downstream of PHYA, PHYB, cryptochromes and yet unidentified UV-B receptors (Ang *et al.*, 1998; Koornneef *et al.*, 1980; Oyama *et al.*, 1997; Ulm *et al.*, 2004). Additionally, HY5 is negatively regulated by the CONSTITUTIVE PHOTOMORPHOGENIC/DEETIOLATED/FUSCA (COP/DET/FUS) protein degradation machinery (Holm *et al.*, 2002; Osterlund *et al.*, 2000; Saijo *et al.*, 2003; Sullivan *et al.*, 2003; Wei und Deng, 2003). HY5 is also thought to act as a signal transducer between hormone and light signaling (Cluis *et al.*, 2004). Recently, the full range of HY5 targets could be revealed, using the ChIP-chip technique (Buck und Lieb, 2004). A total of 3894 binding sites have been discovered, comprising approximately 10% transcription factors and covering more than 60% of the genes early-induced by PHYA or PHYB (Lee *et al.*, 2007). Etiolated seedlings of the *hy5* mutant were grown and analyzed as described above for the other mutants.

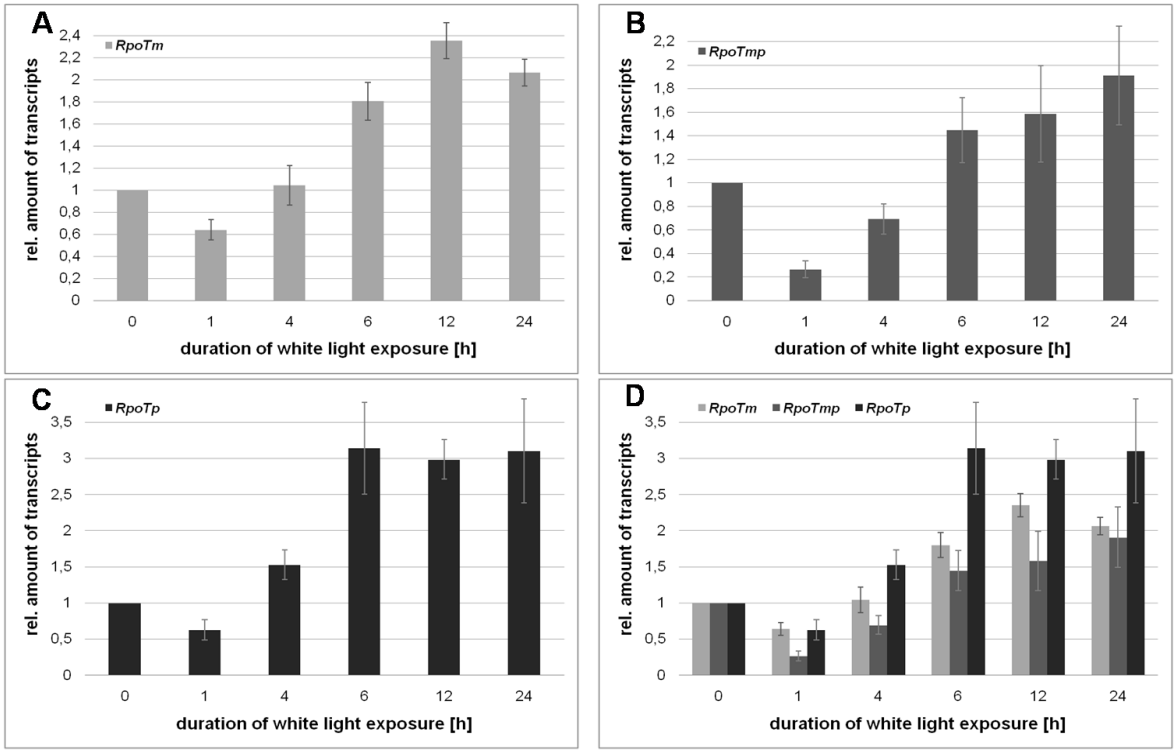


Figure 15: Accumulation of *RpoT* gene transcripts in *hy5* mutants.
Seedlings of *hy5* mutants were grown in darkness for seven days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, four, six, twelve and twenty-four hours of illumination. Relative transcript amounts of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) were determined by quantitative real-time PCR using *UBQ11* mRNA levels as internal standard.

Upon illumination, particularly *RpoTmp* transcript levels decreased quickly. After just one hour a fourfold reduction was observed (Figure 16B). At the same time, transcript abundance of both *RpoTm* (Fig. 15A) and *RpoTp* (Fig. 15C) decreased only slightly to approximately one third of dark control values. In the progress transcript levels of all genes increased. *RpoT* transcripts reached their maximal abundance after six hours of illumination. At that point it was three times higher than in darkened seedlings. Upon further illumination levels did not change anymore. Transcript levels of *RpoTm* showed a steady increase until twelve hours of illumination. However, total increase was only 1.8-fold after six and slightly more than twofold after twelve hours. Levels tended to decrease after twenty-four hours in light. *RpoTmp* transcript levels, which had shown a strong decrease after just one hour, also increased steadily upon exposure to light. While after four hours they did not reach initial levels again, they exceeded dark control values 1.5-fold after six and roughly twofold after twenty-four hours.

Obviously, knockout of *HY5* does not completely inhibit light induced accumulation of *RpoT* transcripts. A net increase in the abundance of mRNAs was found for all three genes.

RpoTp transcript levels increased slightly stronger than those of *RpoTm* and *RpoTmp*. As seen before in other mutants, transcript levels of all genes first declined before increasing upon further illumination.

3.1.6 Expression of *RpoT* genes during *Arabidopsis* leaf development

Expression of the *RpoT* genes in *Arabidopsis thaliana* is most likely not only regulated by light, but by various other internal and external signals. It has previously been shown that the amount of transcripts varied between different tissues (Emanuel *et al.*, 2006). *RpoTp* expression was found to be highest in green tissues, *e.g.* parenchyme cells of leaves, stems and sepals, whereas steady-state transcript levels of *RpoTm* and *RpoTmp* were highest in mitochondria-rich tissues, such as meristems and companion cells flanking the phloem (Emanuel *et al.*, 2006). Additionally, there seemed to be a development-specific regulation of expression. In young seedlings, expression of *RpoTm* and *RpoTmp* was higher than that of *RpoTp*. Transcript levels of the latter started to increase after seven days (Emanuel *et al.*, 2005).

As shown above (3.1.2.1 and 3.1.2.2), expression of all three *RpoT* genes was induced by light even in senescent rosette leaves. Recently, comprehensive data on DNA and RNA metabolism during leaf development were published suggesting differential regulation of expression of plastid genes, but not plastome copy number in cotyledons and leaves of different ages. Both plastid RNA polymerases (NEP and PEP) were shown to transcribe selected genes in young as well as in senescent leaves. However, overall transcriptional activity of plastid genes was reduced in older rosette leaves and PEP was supposed to play a major role at this stage (Zoschke *et al.*, 2007). It was thus very interesting to see if expression of the *RpoT* genes was also regulated during leaf development and if their transcript abundance would play a role in the regulation of organellar transcription. Therefore, *RpoT* gene transcript levels were analyzed in leaf samples ranging from 2-day-old cotyledons to 37-day-old, senescent rosette leaves of *Arabidopsis* (Zoschke *et al.*, 2007).

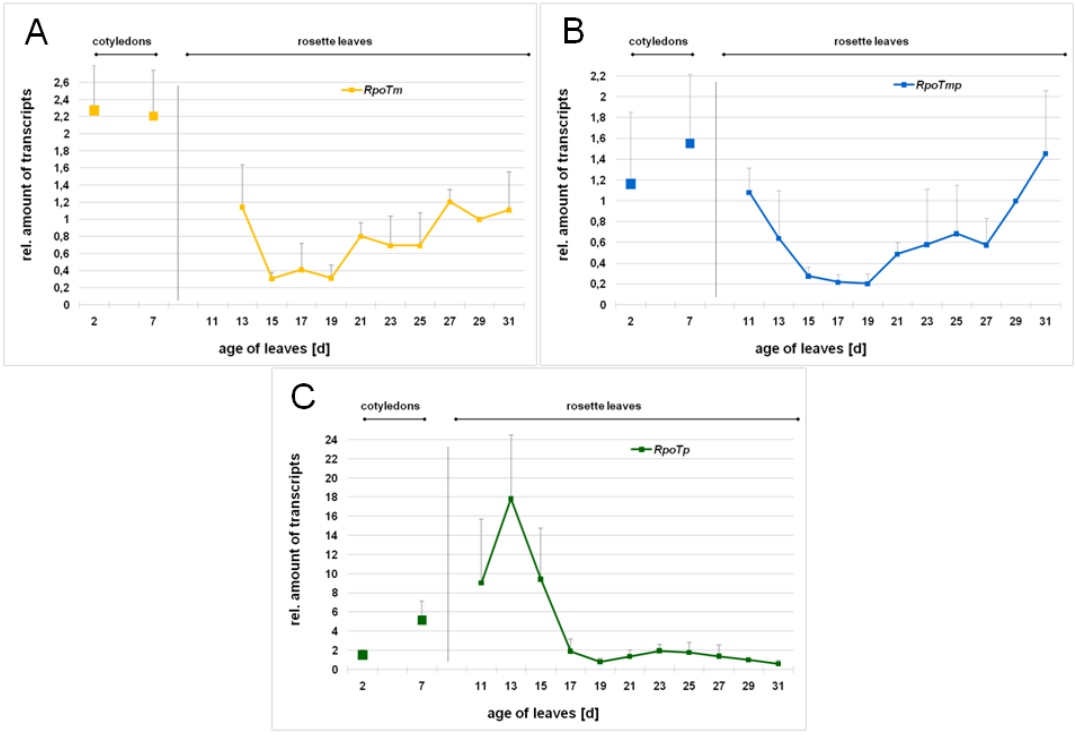


Figure 16: Accumulation of *RpoT* gene transcripts during leaf development.
Cotyledons and rosette leaves of different ages were harvested from 5-, 10-, 40- and 50-day-old *Arabidopsis* plants as described by Zoschke *et al.* (2007). Relative steady-state levels of *RpoTm* (A), *RpoTmp* (B) and *RpoTp* (C) transcripts were measured using quantitative real-time RT-PCR. Relative transcript levels of the nuclear encoded housekeeping gene *UBQ11* were used as internal standard.

As reported similarly by Emanuel *et al.* (2005) transcript levels of *RpoTp* in 2-day-old seedlings were relatively low (Fig. 16C, left side). *RpoTm* and *RpoTmp* transcripts at this stage were more or less equally abundant (Figure 17A and B, left side). In cotyledons of 7-day-old seedlings *RpoTp* expression was increased roughly 2.5-fold, whereas transcript levels of *RpoTm* and *RpoTmp* did not change significantly. In the further progress, steady-state levels of *RpoTp* transcripts steadily increased until reaching a maximum in 13-day-old rosette leaves of 40-day-old plants (Fig. 16C, right side). At that point *RpoTp* values were approximately ninefold higher than in youngest cotyledons. Subsequently, however, mRNA levels decreased rapidly. In 19-day-old leaves they were decreased more than twentyfold. After slightly increasing again in 23-day-old leaves of 50-day-old plants, they further dropped until the leaves got senescent. Transcript levels of *RpoTm* and *RpoTmp* concomitantly declined as leaves were getting older, reaching a minimum between day fifteen and nineteen (Fig. 16A and B, right side). Compared to initial amounts in 2-day-old seedlings there was a sevenfold (*RpoTm*) and fivefold (*RpoTmp*) reduction. Subsequently, transcript levels of both genes steadily increased. In 31-day-old leaves of 50-day-old plants,

the oldest analyzed leaves, which showed obvious signs of senescence, transcripts of *RpoTm* and *RpoTmp* were increased around 3.5- and 7-fold, respectively, compared to their minimal levels.

Overall, expression of *RpoTp* was much higher than that of the other *RpoT* genes in older cotyledons and most notably in young rosette leaves. In older rosette leaves all three *RpoT* genes, although showing broadly different accumulation patterns were expressed at similar levels. While *RpoTp* transcripts declined in parallel with overall plastid transcription (Zoschke *et al.*, 2007), *RpoTm* and *RpoTmp* mRNAs accumulated in senescent leaves. The results suggest a pivotal role for RpoTp in older cotyledons and young rosette leaves, whereas its importance seems to decrease in older leaves. In very young cotyledons *RpoTp* transcripts are, however, less abundant, suggesting that PEP is more active at that time.

3.2 Analysis of organellar gene copy numbers and transcript accumulation in chlorophyll-deficient *Arabidopsis* seedlings

3.2.1 Light- induced steady-state transcript accumulation of two plastidial genes in green and white seedlings of *Arabidopsis*

Higher plant plastids harbor two types of RNA polymerases. Besides the nuclear encoded phage-type enzymes RpoTp and RpoTmp (NEP; (Hess und Börner, 1999; Liere und Börner, 2007) there is a second, plastid encoded polymerase of the eubacterial type (PEP; (Liere und Börner, 2007; Shiina *et al.*, 2005). To date, it has not been possible to isolate mutants or transplastomic *Arabidopsis* plants lacking PEP activity. However, by growing *Arabidopsis* on medium containing spectinomycin, an inhibitor of plastid translation (Moazed und Noller, 1987; Wallace *et al.*, 1974), seedlings exhibit a white leaf phenotype (Fig. 17) and possess immature, photosynthetically inactive chloroplasts (Hess *et al.*, 1994; Svab *et al.*, 1990; Swiatecka-Hagenbruch *et al.*, 2008; Zubko und Day, 1998).

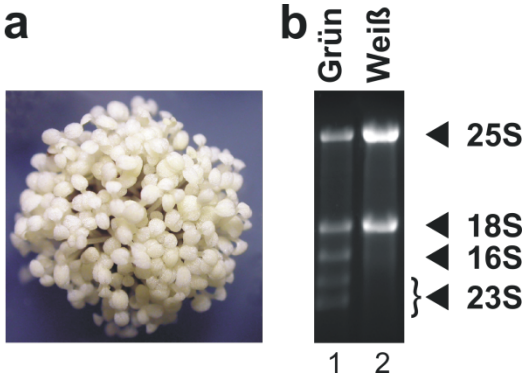


Figure 17: Chlorophyll-deficient *Arabidopsis thaliana* seedlings.
Seeds were sown on SEA-medium containing the antibiotic spectinomycin. Seedlings develop white cotyledons (a). RNA isolated from 21-day-old plants showed a drastic reduction of ribosomal RNAs (b, lane 2) compared to RNA from green tissues of the same age (b, lane 1). Figure taken from Swiatecka-Hagenbruch (2008).

It has recently been shown that transcript levels of plastidial, mitochondrial and nuclear genes in chlorophyll-deficient seedlings of *Arabidopsis thaliana* differ broadly from those in normal green seedlings (Swiatecka-Hagenbruch, 2008; Swiatecka-Hagenbruch *et al.*, 2007). Transcripts of all three *RpoT* genes accumulate to higher levels in white seedlings. Additionally, many plastidial genes show significantly lower expression and a lot of mitochondrial transcripts in contrast show higher steady-state levels. Furthermore, transcription rates of selected plastid genes were shown to be induced by light using primer

extension assays (Swiatecka-Hagenbruch, 2008). Promoter usage also differs for some plastid genes between green and white seedlings (Swiatecka-Hagenbruch, 2008).

In order to further analyze the influence of PEP on transcription in chloroplasts, expression of two plastid genes, *clpP* and *rrn16*, was studied using quantitative real-time PCR. These genes were chosen, because they both possess two transcription initiation sites. *clpP* is transcribed from a strong type-II NEP-promoter (P*clpP*-58) and a weak PEP promoter (P*clpP*-115), whereas *rrn16* is transcribed from the weak Pc-promoter P*rrn16*-139 and the strong PEP promoter P*rrn16*-112 (Sriraman *et al.*, 1998; Sriraman *et al.*, 1998; Swiatecka-Hagenbruch, 2008; Vera und Sugiura, 1995).

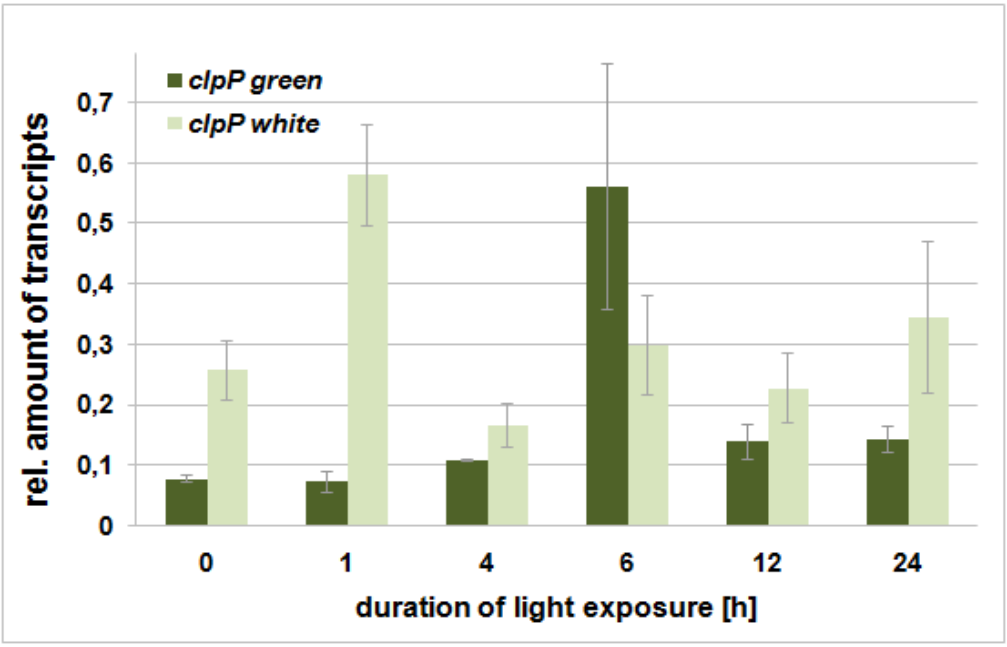


Figure 18: Accumulation of *clpP* gene transcripts in green and white *Arabidopsis* seedlings. Wildtype (Col-0) seedlings were grown in darkness for 7 days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, six and twelve and twenty-four hours of illumination. To get chlorophyll-deficient *Arabidopsis*, seeds were sown on medium containing spectinomycin. Relative steady-state levels of *clpP* transcripts from green (dark green bars) and white seedlings (light green bars) were measured using quantitative real-time RT-PCR. Relative transcript levels of the nuclear encoded housekeeping gene *UBQ11* were used as internal standard.

Steady-state transcript levels of *clpP* in normal, green seedlings did not change significantly in the first four hours of exposure to white light. However, after six hours, they were increased approximately sevenfold (Fig. 18; dark green bars). Twelve hours after illumination, mRNA levels were strongly decreased again and did not change any further. After twenty-four hours, transcript levels were less than twofold higher, compared to initial levels in etiolated seedlings.

The expression pattern in chlorophyll-deficient seedlings looked completely different. In the control samples harvested in darkness (0) *clpP* transcript levels were three times higher than in seedlings grown without spectinomycin. After only one hour of illumination, there was a twofold increase (Fig. 18; light green bars). At that point, transcript levels in white seedlings were more than seven times higher than in green seedlings under the same conditions. Three hours later *clpP* expression in white seedlings dropped drastically to less than one third, compared to values after one hour. Subsequently, transcript levels slightly increased again but did not change significantly until twenty-four hours of illumination although showing some variation.

Thus, there was a dramatic difference in the expression of *clpP* between normal, green seedlings and spectinomycin-treated, white seedlings. The lack of PEP obviously had a major impact on transcript accumulation, although the NEP-promoter is supposed to be much stronger than the PEP-promoter.

The *rrn16* gene in *Arabidopsis* is transcribed from a strong PEP-promoter and a weak Pc-promoter. In etiolated seedlings grown on medium without any special treatment, transcript levels slightly increased after one hour of illumination, followed by a pronounced decrease after four hours (Fig. 19; dark green bars). As observed similarly for *clpP*, the amount of transcripts after six hours was increased substantially to more than elevenfold. In the progress levels dropped again to roughly one third of the maximum.

As expected, the expression pattern in white seedlings differed fundamentally (Fig. 19; light green bars). Transcript levels in the dark were markedly higher in spectinomycin-treated compared to untreated seedlings. Upon illumination the amount of transcripts rapidly decreased to a tenth after just one hour. In the next few hours there was no significant change, but after twelve hours of illumination transcript levels showed another increase, leading to threefold higher values. After twenty-four hours the amount of mRNAs declined again.

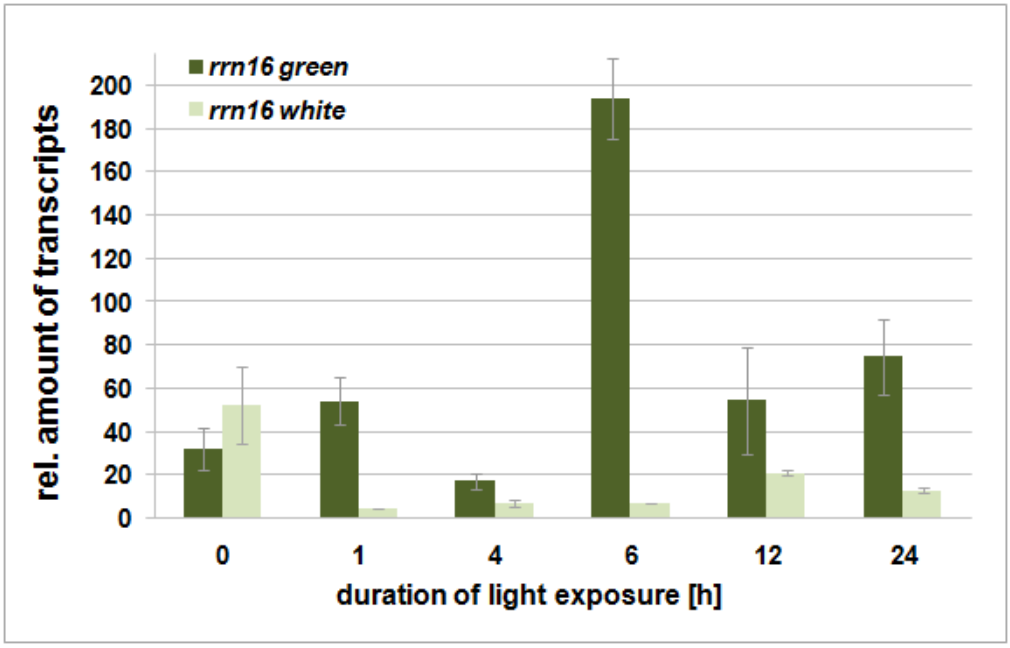


Figure 19: Accumulation of *rrn16* gene transcripts in green and white *Arabidopsis* seedlings. Wildtype (Col-0) seedlings were grown in darkness for 7 days and subsequently exposed to white light. Samples were taken in darkness (0) and after one, six and twelve and twenty-four hours of illumination. To get chlorophyll-deficient *Arabidopsis*, seeds were sown on medium containing spectinomycin. Relative steady-state levels of *rrn16* transcripts from green (dark green bars) and white seedlings (light green bars) were measured using quantitative real-time RT-PCR. Relative transcript levels of the nuclear encoded housekeeping gene *UBQ11* were used as internal standard.

The experiments revealed that *clpP* as well as *rrn16* transcript accumulation in normal, 7-day-old etiolated wildtype seedlings is induced by white light. Most obviously, both genes showed a significant increase in expression six hours after illumination. In spectinomycin-treated seedlings grown under the same conditions transcript accumulation differed substantially. In the case of *clpP* induction took place much earlier, after just one hour. By contrast, *rrn16* transcript accumulation reached its maximum later, after twelve hours. Overall, a tendency towards higher transcript levels in white compared to green seedlings was observed for *clpP*, whereas *rrn16* transcripts accumulated to considerably lower levels in white plants.

3.2.2 Mitochondrial transcript levels in photosynthetically inactive, white *Arabidopsis* seedlings

It has previously been shown that the developmental status of plastids strongly influences the expression of genes encoded in the nucleus and mitochondria. In white leaves of the barley *albostrians* mutant transcript levels of selected mitochondrial genes as well as the

RpoT genes were found to be increased (Emanuel *et al.*, 2004; Hedtke *et al.*, 1999). The undeveloped plastids of this mutant lack ribosomes, photosynthetic activity and have only trace amounts of chlorophyll (Hess *et al.*, 1993; Hess *et al.*, 1994; Hess *et al.*, 1992).

Growing *Arabidopsis* on medium containing spectinomycin also gives rise to white, chlorophyll-deficient plants lacking the ability to perform photosynthesis (Svab *et al.*, 1990; Zubko und Day, 1998). It has been shown recently that these plants show elevated levels of *RpoT* gene transcripts (Swiatecka-Hagenbruch, 2008). Using microarrays, the same study revealed that expression of several mitochondrial genes is upregulated in white seedlings (Swiatecka-Hagenbruch, 2008).

In order to further investigate the influence of impaired photosynthesis and absence of PEP on mitochondrial transcription, four representative mitochondrial genes were analyzed by quantitative real-time PCR in spectinomycin-treated and untreated wildtype *Arabidopsis* seedlings. Four genes representing the bandwidth of mitochondrial functions were chosen, *atp1*, *cox1*, *rps4* and *nad6*, coding for ATPase, cytochrome c oxidase, ribosomal protein and NADH dehydrogenase subunits. Plants were grown on SEA-medium with and without spectinomycin and harvested after ten and twenty days.

Figure 21 shows the results of the analyses. In each graph (A,B,C and D) the two bars on the left represent the relative amount of transcripts found in 10- and 20-day-old green seedlings, the two bars on the right those found in chlorophyll-deficient seedlings of the same ages. In green seedlings, transcript levels tended to decrease slightly from 10- to 20-day-old plants, whereas in 20-day-old spectinomycin-treated plants a tendency towards higher levels could be observed for *cox1* and *nad6* transcripts. Relative amounts of *atp1* and *rps4* transcripts in 10- and 20-day-old white samples did not differ.

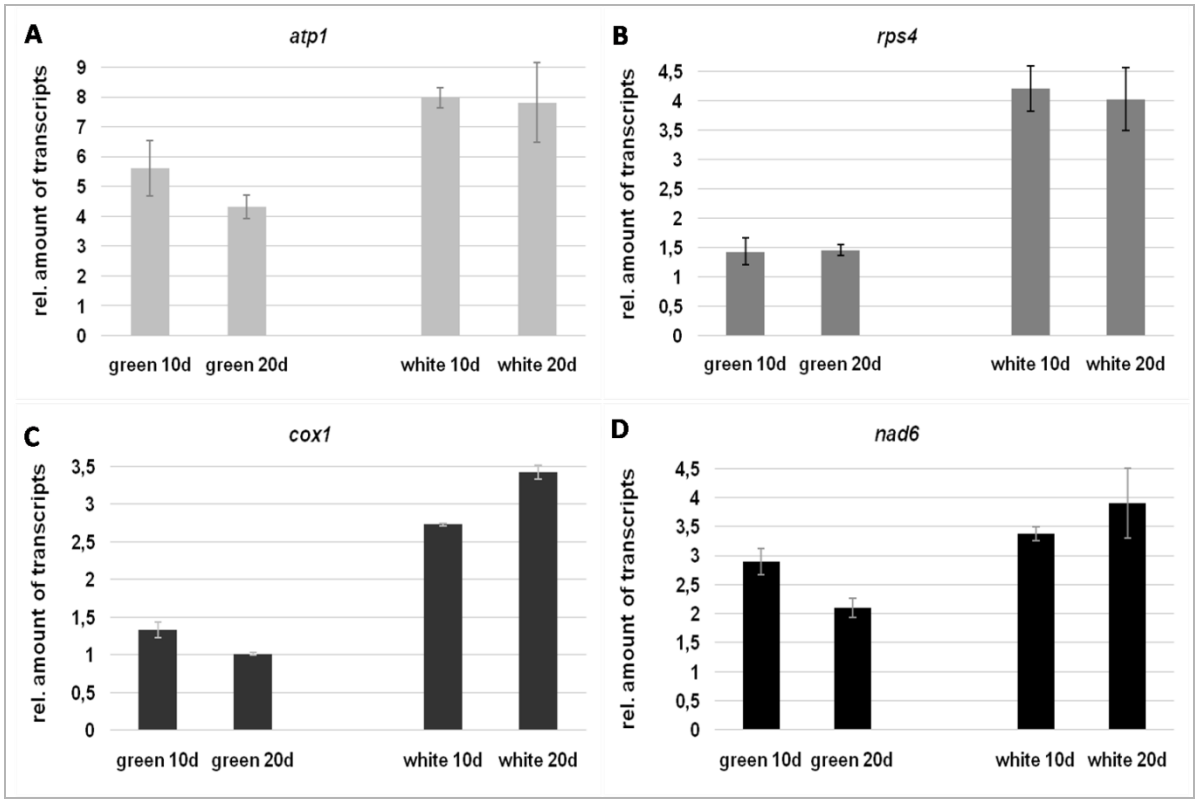


Figure 20: Accumulation of mitochondrial gene transcripts in green and white *Arabidopsis* seedlings.
Wildtype (Col-0) seedlings were grown on SEA-medium. To get chlorophyll-deficient plants, seeds were sown on medium containing spectinomycin. Seedlings were grown under white light in a long-day regime and harvested after 10 and 20 days. Relative steady-state levels of *atp1* (A), *cox1* (B), *rps4* (C) and *nad6* (D) were measured using quantitative real-time RT-PCR. Relative transcript levels of the nuclear encoded housekeeping gene *UBQ11* were used as internal standard.

It became obvious that transcript levels of all analyzed genes were significantly increased in white, photosynthetically inactive seedlings. The most decided increase was observed for *rps4* transcripts (3-fold in 10-, 2.8-fold in 20-day-old samples; Fig. 20C); whereas the smallest changes were found for *nad6* transcripts (1.2-fold in 10-, 1.9-fold in 20-day-old plants; Fig. 20D). Transcripts of *atp1* were increased 1.4-fold in 10- and 1.8-fold in 20-day-old white compared to green seedlings (Fig. 20A) and in the case of *cox1* the amount of transcripts in 10- and 20-day-old white samples was two- and 3.3-fold higher, respectively.

Hence, it could be approved that photosynthetic inactivity in plants lacking proper plastid translation and PEP machinery directly influences steady-state transcript levels of mitochondrial genes.

3.2.3 Endopolyploidy and mitochondrial gene copy numbers in photosynthetically inactive, white *Arabidopsis* seedlings

Hedtke *et al.* (1999) showed that in addition to an increase in mitochondrial transcript levels copy numbers of mitochondrial genes were elevated in *albostrians* mutants of barley (Hedtke *et al.*, 1999). Increased expression of mitochondrial genes was also shown for chlorophyll-deficient, white *Arabidopsis* (see 3.2.2) and albino mutants of rye (Ballesteros *et al.*, 2009). To get a deeper insight into dimensions and complexity of the influence of the plastids' developmental status on mitochondria, copy numbers of mitochondrial genes were determined in green and white seedlings of *Arabidopsis*. Experimental and growth conditions were the same as described in chapter 3.2.2; samples were analyzed from 10- and 20-day-old seedlings. To be able to correctly calculate copy numbers per cell using quantitative real-time PCR, we determined a possible effect of spectinomycin treatment on polyploidization of the nuclear genome. Flow-cytometric analyses revealed slightly increased nuclear endopolyploidization levels in white, spectinomycin-treated seedlings in comparison to green, untreated plants (Fig. 21). The average number of nuclear genome copies per cell (mean C-value; (Zoschke *et al.*, 2007) in green seedlings was 4.9 and 4.4 after ten and twenty days, respectively. In white plants the mean C-value was roughly six for both age stages.

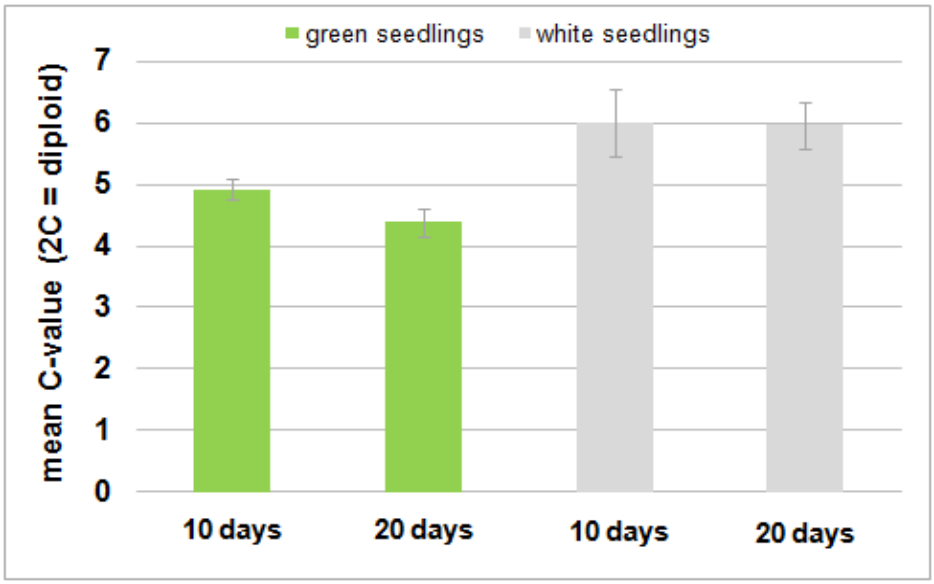


Figure 21: Nuclear endopolyploidization in green and white *Arabidopsis* seedlings.
Wildtype (Col-0) seedlings were grown on SEA-medium. To get chlorophyll-deficient plants, seeds were sown on medium containing spectinomycin. Seedlings were grown under white light in a long-day regime and harvested after ten and twenty days. Mean C-values were determined flow-cytometrically as described in chapter II.2.10. Endopolyploidization levels were calculated for nuclei of 10- and 20-day-old white (gray bars) and green seedlings (green bars) of *Arabidopsis*.

Comparison of the four abovementioned mitochondrial genes (see 3.2.2) in untreated and chlorophyll-deficient seedlings by quantitative real-time PCR showed that the copy numbers of all analyzed genes increased slightly from ten to twenty days in green seedlings, whereas they decreased in white plants (Fig. 22). In comparison to untreated seedlings, however, white seedlings generally contained significantly more gene copies per cell. After ten days gene copy numbers were 3.4 to 4.3 times higher in white compared to green seedlings and after twenty days they were still 2.2 to 2.5 times higher.

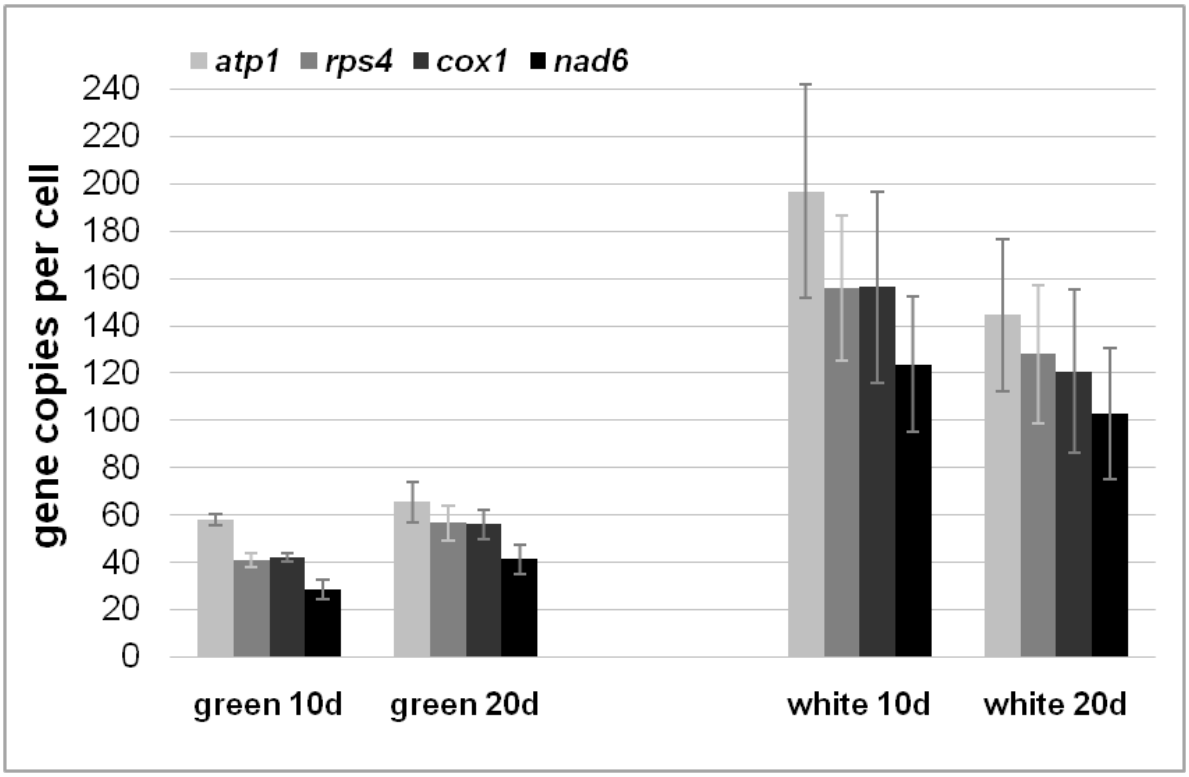


Figure 22: Mitochondrial gene copy numbers in green and white *Arabidopsis* seedlings.
Relative gene copy numbers of four mitochondrial genes were measured in 10- and 20-day-old seedlings of *Arabidopsis* by quantitative real-time RT-PCR. White, spectinomycin-treated (right side) and normal green, untreated seedlings (left side) were analyzed. The nuclear encoded single-copy gene *RpoTp* was used as internal standard. Total number of gene copies per cell was then calculated as described in chapter 2.2.6 using the relative gene copy number, the mean C-value and the number of NUMTs.

Thus, the developmental status of plastids seems to have a distinctive effect on mitochondrial gene copy numbers also in photosynthetically inactive *Arabidopsis thaliana*. This supports the theory of elevated overall mitochondrial activity in plants lacking the ability to produce energy by performing photosynthesis. An influence of plastid status on mitochondrial metabolism in that peculiarity necessitates a highly complex network of cell compartments, involving plastids, mitochondria and the nucleus.

3.3 Changes in mitochondrial gene copy numbers and transcript levels during leaf development in *Arabidopsis thaliana*

3.3.1 Analyses of mitochondrial gene copy numbers during leaf development

In the present study it was shown that the developmental status of plastids and photosynthetic activity strongly influence mitochondrial RNA and DNA metabolism (see 3.2.2 and 3.2.3). Gene copy numbers as well as transcript levels differed markedly between green and white seedlings of *Arabidopsis*. They also seemed to vary between 10- and 20-day-old plants. Recently, Zoschke *et al.* (2007) showed that plastome copy numbers in *Arabidopsis* varied from about 1000 to 1700 per cell, without a significant in- or decreasing trend during development from young cotyledons to old rosette leaves (Zoschke *et al.*, 2007). To further investigate DNA amounts of mitochondrial genes during leaf development, four representative genes, *atp1, rps4, cox1* and *nad6* were chosen as targets for quantitative real-time PCR analyses. The selected genes not only represent the variety of mitochondrial functions, but also include a pair of adjacent genes (*nad6* and *rps4*), making it possible to further study the behavior of subgenomic molecules. The nuclear encoded gene *RpoTp* was chosen as an internal standard. *RpoTp* encodes a plastid targeted RNA polymerase of the phage type (Hess und Börner, 1999; Lerbs-Mache, 1993; Liere und Börner, 2007) and is a single-copy gene, therefore being beneficial for the determination of unknown gene copy numbers. Because extensive endopolyploidization in nuclei of *Arabidopsis* leaves during leaf development has been shown (Zoschke *et al.*, 2007), the same DNA samples as described in Zoschke *et al.* (2007) were used for the determination of mitochondrial gene copy numbers, which were corrected accordingly. To this end, total gene copy numbers per cell were calculated as the product of the $2^{(-\Delta C_T)}$ -values (the relative gene copy numbers) the mean C-value (Zoschke *et al.*, 2007) and the number of nuclear copies of mitochondrial genes (NUMTs; (Lopez *et al.*, 1994; Richly und Leister, 2004).

In contrast to copy numbers of plastid genes, considerable differences were found between the copy numbers of individual mitochondrial genes (Fig. 23). Most strikingly, *atp1* showed highest copy numbers in all analyzed samples except for youngest cotyledons. As expected, the amount of gene copies of the adjacent genes *nad6* and *rps4* did not show significant differences. To exclude unequal PCR efficiencies of the individual assays, fragments of three of the analyzed genes were cloned into pBluescript® II SK (+) and the vector DNA was subsequently used as a template for real-time PCR. An equal ratio of amplification rates between all analyzed genes was found, *i.e.* PCR efficiencies were

identical for the assays (Fig. 24). Hence, the differences in gene copy numbers are most probably due to the existence of different subgenomic molecules carrying the genes. Whereas *nad6* and *rps4* are suggested to almost exclusively reside on the same molecule, *atp1* is obviously located on a separate chromosome frequently.

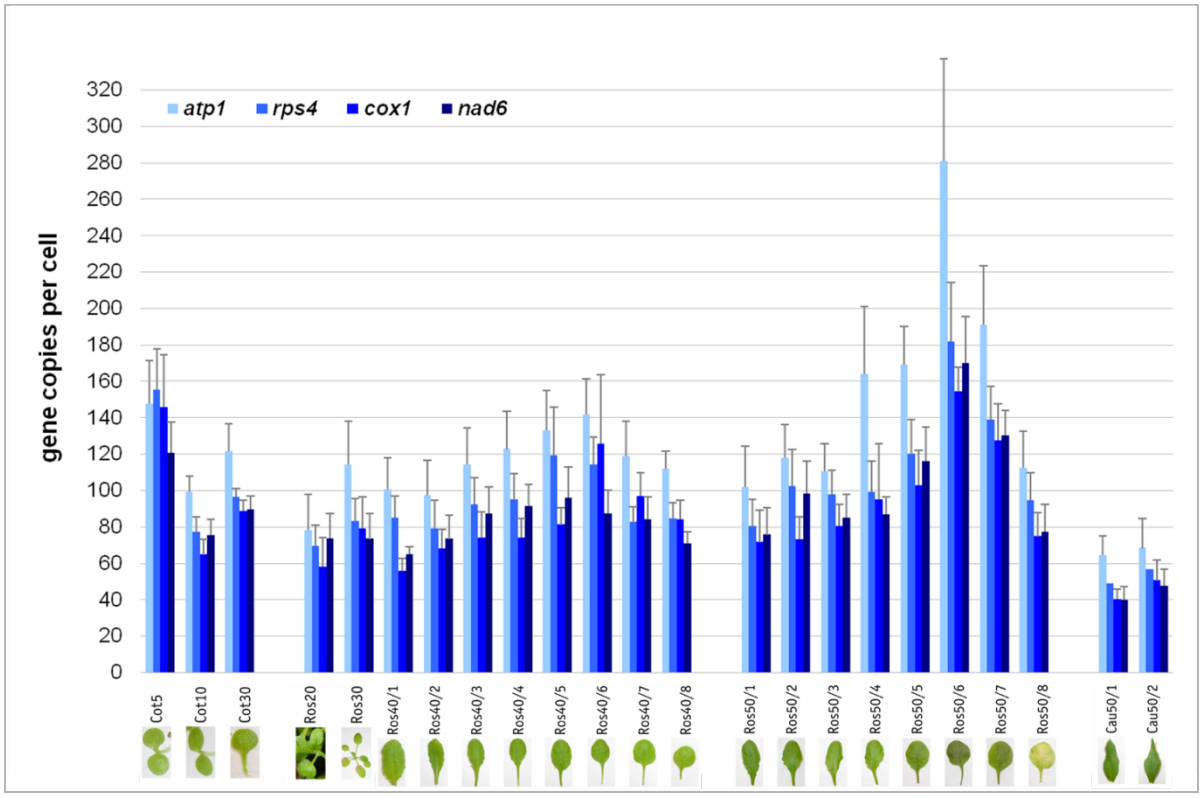


Figure 23: Copy numbers of mitochondrial genes during leaf development in *Arabidopsis*. Relative gene copy numbers of four mitochondrial genes, *atp1*, *rps4*, *cox1* and *nad6*, were measured in leaves of different ages using quantitative real-time PCR. The nuclear encoded single-copy gene *RpoTp* was used as internal standard. Total number of gene copies per cell was then calculated as outlined above using the relative gene copy number, the mean C-value and the number of NUMTs. Denomination of the different leaf age stages is defined in Zoschke *et al.* (2007).

The observed values for total gene copy numbers varied from a minimum of about 40 in cauline leaves to a maximum of around 280 (*atp1*, Ros50/6). For the most part, between 50 and 150 copies per cell were determined (Fig. 23). These numbers are decidedly smaller than the estimated average number of discrete mitochondria per cell. Even though mitochondrial quantity most likely differs between individual cell types, it is assumed that *Arabidopsis* mesophyll cells contain around 600 or more discrete mitochondria, depending on the physiological status of the cell (Sheahan *et al.*, 2005; Stickens und Verbelen, 1996). Similarly, in tobacco and *Medicago truncatula* mesophyll protoplasts, an average of 500-600 mitochondria per cell was monitored (Sheahan *et al.*, 2004). Therefore, the findings

suggest that most, if not all, mitochondria in *Arabidopsis* leaf cells contain an incomplete chondrome.

Gene copy numbers of all analyzed genes showed a pronounced increase in older rosette leaves (Fig. 23). This increase occurred in 27- to 31-day-old leaves (Ros50/4 - Ros50/6). As leaves started to bleach after day 32 (Ros50/7) the number of gene copies rapidly declined again. Thus, these results suggest mitochondrial gene copy numbers to be closely associated with the developmental status of leaves and the whole plant.

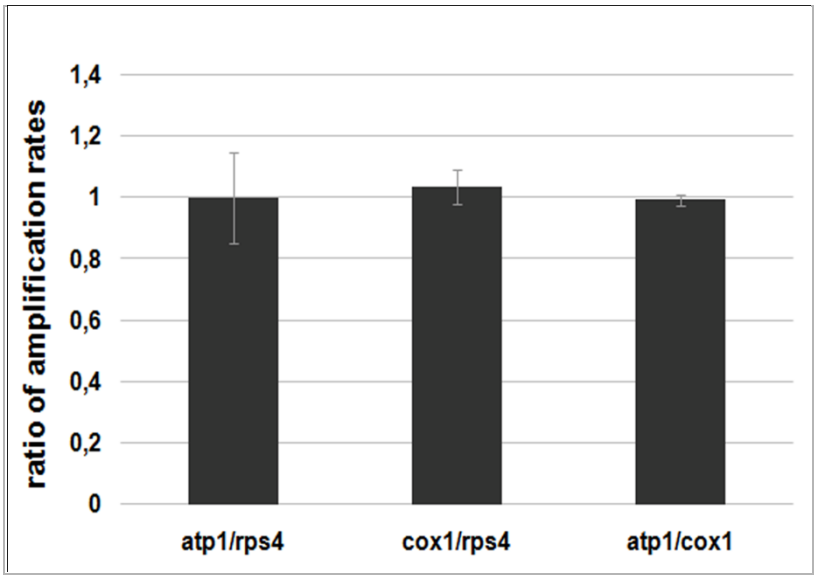


Figure 24: Comparison of the amplification rate of different mitochondrial gene assays used for quantitative real-time PCR.
Fragments of the genes used in the analyses described above were cloned into pBluescript® II SK (+) (Fermentas). Vector DNA was then used as a template for qPCR. A ratio of 1 between the amplification rates of two distinct genes means that PCR efficiency is identical. As *nad6* and *rps4* gene copy numbers did not differ significantly, only *rps4* was tested here.

3.3.2 Steady-state transcript levels of mitochondrial genes during leaf development

It remains unknown in how far, and if at all, changes in mitochondrial gene copy numbers have a regulatory effect, *e.g.* by affecting downstream processes like transcription and translation. If the availability of more gene copies would directly lead to an increased availability of the corresponding gene product, regulation on the DNA level could play an important role in the modulation of metabolic and developmental processes as well as in various cell responses.

To investigate steady-state levels of mitochondrial genes during leaf development in *Arabidopsis*, quantitative real-time RT-PCR experiments with total cDNA from leaves of different ages were carried out. As the mitochondrial genes analyzed in this study do not

contain introns (Unseld *et al.*, 1997) the primers designed for the analyses of gene copy numbers were used again for this purpose. Due to its stable expression throughout the different developmental stages, *UBQ11* was used as an internal standard.

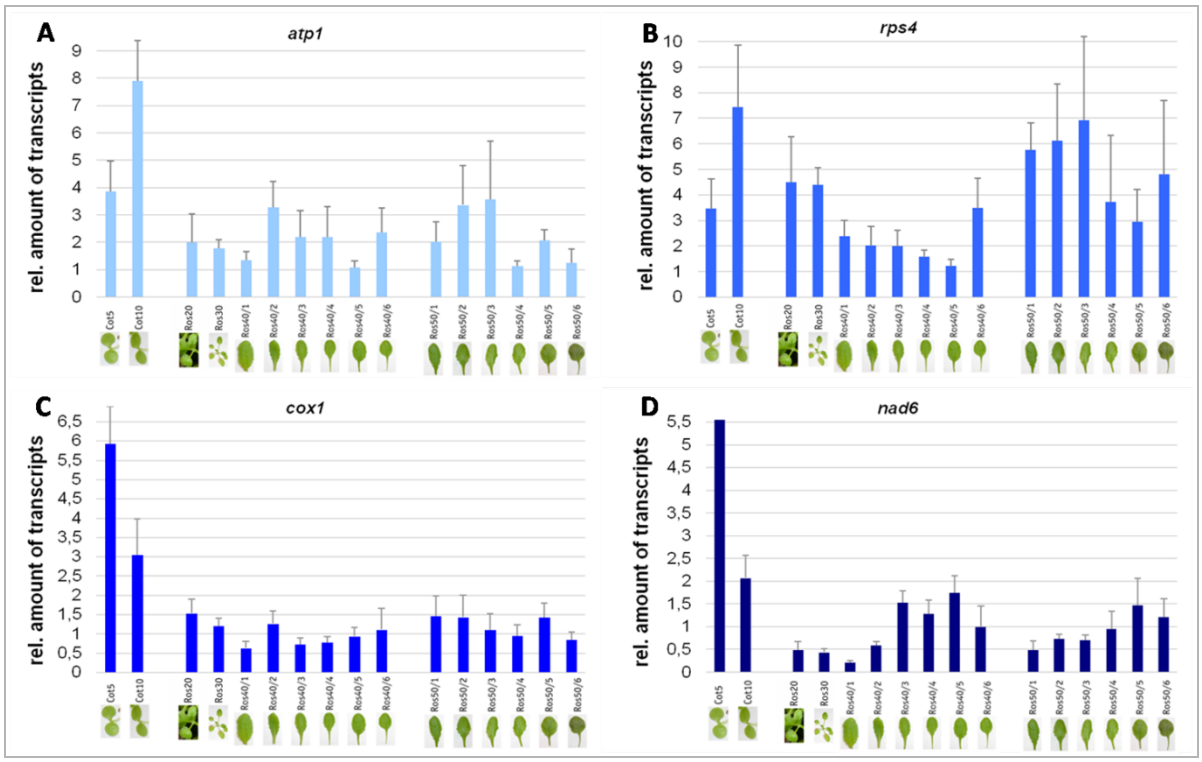


Figure 25: Steady-state transcript levels of mitochondrial genes during leaf development in *Arabidopsis*.
Relative amounts of transcripts of four mitochondrial genes, *atp1*, *rps4*, *cox1* and *nad6*, were measured using quantitative real-time RT-PCR. The relative transcript level of the nuclear encoded housekeeping gene *UBQ11* was used as internal standard. Denomination of the different leaf age stages is defined in Zoschke *et al.* (2007).

Transcripts of *cox1* and *nad6* genes showed highest steady-state levels in 5-day-old cotyledons (Cot5; Fig. 25C and D). The amount of transcripts then declined rapidly to around one half in 10-day-old cotyledons (Cot10) and even further in young rosette leaves, reaching a minimum at stage Ros40/1 (11-day-old leaves). In the progress, *cox1* transcripts showed relatively stable abundance with a slight increase towards stage Ros50/1 (21-day-old leaves) and a slight decrease in the oldest leaves analyzed (Ros50/6). Transcript levels of *nad6* increased considerably in stages Ros40/3 to Ros40/5 (15- to 19-day-old leaves), before dropping again until Ros50/1 (21-day-old leaves). Subsequently, there was an almost 2.5-fold increase towards Ros50/5 (29-day-old leaves) followed by a decrease in the oldest analyzed leaves (Ros50/6).

Transcripts of *atp1* also showed highest levels in cotyledons (Fig. 25A). However, unlike *cox1* and *nad6*, *atp1* mRNA was found to be most abundant in stage Cot10 (10-day-old cotyledons), showing twice as high values as in stage Cot5 (5-day-old cotyledons). In rosette leaves, the amount of *atp1* transcripts, although showing pronounced variations, did not reveal any obvious tendencies apart from a slight increase from stage Ros50/1 (21-day-old leaves) to stage Ros50/3 (24-day-old leaves) and a significant decline in the oldest analyzed leaves.

In the case of *rps4*, the amount of transcripts in cotyledons was not significantly higher than in young rosette leaves (Fig. 25B). Nevertheless, mRNAs showed highest abundance in stage Cot10. In 5-day-old cotyledons, only approximately half the amount was detected. In the progress, transcript levels continuously declined until reaching a minimum in 19-day-old leaves (Ros40/5). Subsequently, they increased again until stage Ros50/3 (25-day-old leaves) and finally decreased in the oldest leaves.

Thus, transcript abundance of mitochondrial genes changes to some extent during leaf development, tending to be higher in cotyledons compared to mature rosette leaves. However, the increased gene copy numbers which had been found in older leaves (Fig. 23) were not reflected by any of the investigated genes' steady-state transcript levels.

3.3.3 Oxygen consumption the development of *Arabidopsis* cotyledons and leaves

It has been shown previously that mitochondrial gene copy numbers vary to a great extent during plant and leaf development (see 3.3.1). Especially during early leaf senescence, copy numbers of all analyzed genes increased drastically. The same phenomenon was observed in seedlings that had been treated with spectinomycin (see 3.3.2). These plants are chlorophyll-deficient and photosynthetically inactive. In the seedlings, transcript levels were also increased suggesting a generally upregulated mitochondrial activity. However, no elevated steady-state transcript levels could be observed during early leaf senescence.

The major function of mitochondria is the synthesis of ATP by oxidative phosphorylation (Saraste, 1999). This respiration can be measured by monitoring the O_2-consumption of respective tissues. An increased respiration would lead to more ATP production and would therefore be an indication of energy consuming processes. A correlation between respiration activity and mitochondrial genome copy numbers has been shown earlier in germinating cotyledons of peanut (Breidenbach *et al.*, 1967).

To further analyze the increase of gene copy numbers in rosette leaves during early senescence, O_2-consumption was measured in leaves of different ages. To this end, cotyledons from 5- and 10-day-old seedlings, as well as young, middle-aged and older leaves in the stage of early senescence from 40- and 50-day-old plants were sampled. Parallel to the monitoring of respiration, endopolyploidy levels (Fig. 26) and gene copy numbers of four mitochondrial genes (Fig. 26) were determined.

As expected, endopolyploidization increased with the age of the leaves (Fig. 26), showing similar values as reported by Zoschke *et al.* (2007). Copy numbers of *atp1*, *rps4*, *cox1* and *nad6* also increased from young to old rosette leaves (Fig. 28). Total numbers slightly differed from those shown in Figure 24 due to different growth conditions and choice of leaves. The characteristic increase in leaves during early senescence, however, could be approved.

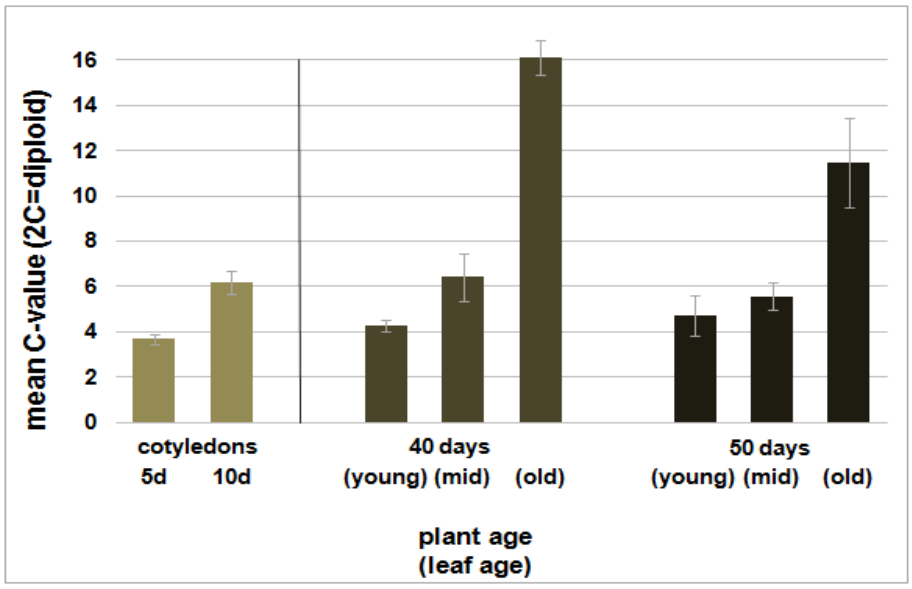


Figure 26: Changes in nuclear endopolyploidization during development of *Arabidopsis* leaves. Wildtype (Col-0) seedlings were grown on soil/vermiculite under long day conditions. Mean C-values were determined flow-cytometrically as described in chapter 2.2.10. Endopolyploidization levels were calculated for nuclei of cotyledons from 5- and 10-day-old seedlings (light brown bars, left side) and rosette leaves of different ages from 40- and 50-day-old plants.

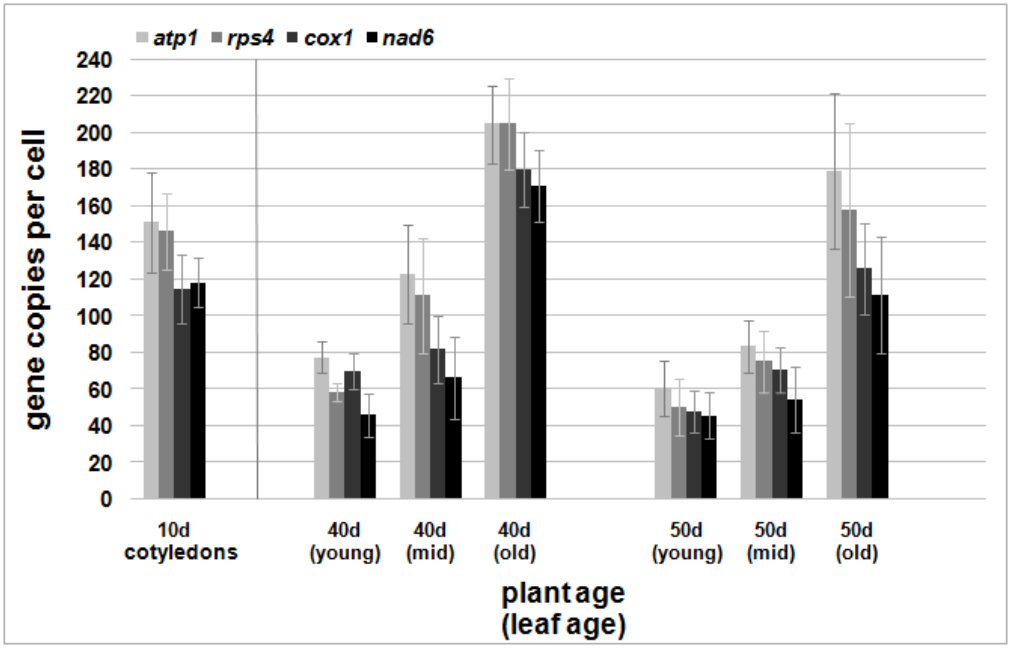


Figure 27: Mitochondrial gene copy numbers in leaves of different ages.
Relative gene copy numbers of four mitochondrial genes, *atp1*, *rps4*, *cox1* and *nad6*, were measured in leaves of different ages using quantitative real-time PCR. The nuclear encoded single-copy gene *RpoTp* was used as internal standard. Total number of gene copies per cell was then calculated as outlined above using the relative gene copy number, the mean C-value and the number of NUMTs. Denomination of the different leaf age stages is defined in Zoschke *et al.* (2007).

Respiration activity was highest in very young cotyledons of 5-day-old plants (Fig. 28). In cotyledons of 10-day-old seedlings oxygen-consumption dropped to roughly one half. In 40-, as well as in 50-day-old plants respiration activity was strongest in young rosette leaves and declined continuously as leaves grew older. Lowest activity was detected in rosette leaves showing signs of early senescence (Fig. 28; dark green bars). In young rosette leaves of 40-day-old plants, O_2-consumption was more than twice as high as in old leaves from the same plants. In old leaves of 50-day-old plants respiration was slightly less than half compared to young rosette leaves. Overall oxygen consumption was lower in 50-day-old plants. Hence, these findings suggest a negative correlation between leaf age as well as mitochondrial gene copy numbers and respiration activity in *Arabidopsis thaliana*.

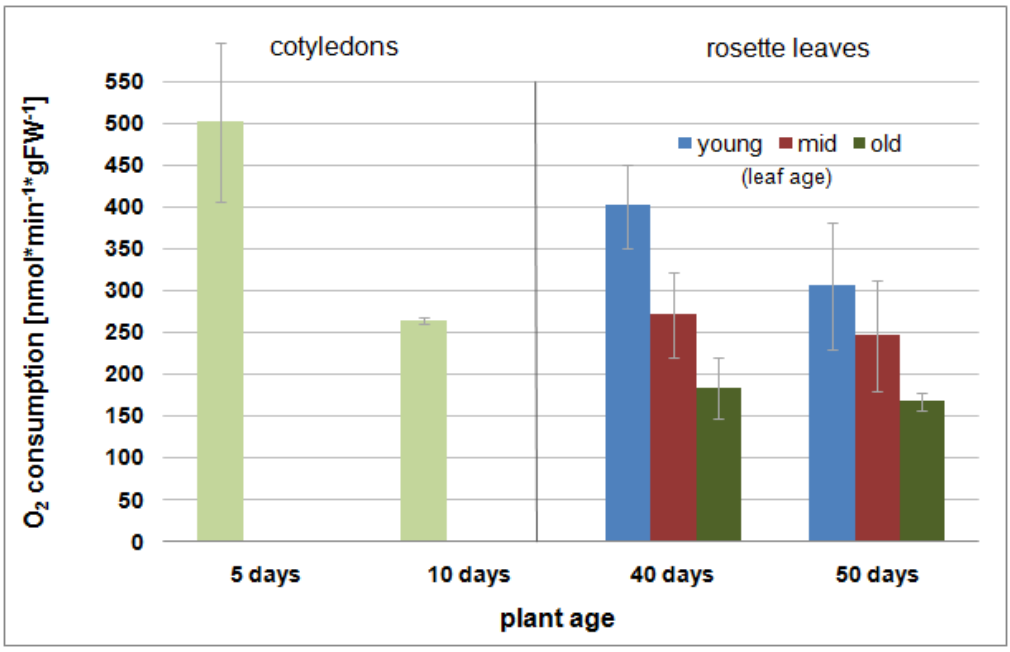


Figure 28: Respiration in cotyledons and leaves of different ages.
O_2-consumption of cotyledons (light green bars), young (blue bars), middle-aged (red bars) and old rosette leaves (dark green bars) was measured as described in chapter 2.2.11.

4 Discussion

4.1 Analysis of light-induced regulation of *RpoT* gene expression in *Arabidopsis thaliana* seedlings and mature rosette leaves

Light is one of the most important environmental factors and plays a major role in life and development of almost every higher plant species. As sessile organisms, plants have to be able to constantly monitor and respond to their light environment. Light quality, intensity, direction and periodicity are sensed through different classes of photoreceptors, such as the red/far-red light absorbing phytochromes and the UV-A/blue-light absorbing cryptochromes (Chen *et al.*, 2004). Absorption of light by those specialized receptor proteins leads to the activation of a variety of signaling cascades that trigger specific responses on physiological and molecular levels including fundamental processes like seed germination (Hennig *et al.*, 2002; Reed und Chory, 1994; Shinomura *et al.*, 1994; Shinomura *et al.*, 1996), de-etiolation or photomorphogenesis (Lin, 2002; Lin *et al.*, 1998; Mazzella *et al.*, 2001; Whitelam *et al.*, 1998), the shade avoidance syndrome (Franklin, 2008; Lorrain *et al.*, 2006; Smith und Whitelam, 1997), induction of flowering (Bagnall *et al.*, 1995; Bagnall *et al.*, 1996; El-Din El-Assal *et al.*, 2003; Guo *et al.*, 1998; Mockler *et al.*, 1999) and many more.

Plastids are the sites of photosynthesis and it is thus not surprising that the expression of many plastid genes is regulated in a light-dependent manner (Christopher und Mullet, 1994; Link, 1996; Mayfield *et al.*, 1995; Mochizuki *et al.*, 2004; Pfannschmidt *et al.*, 1999; Pfannschmidt *et al.*, 1999; Rapp *et al.*, 1992; Tsunoyama *et al.*, 2004; Tsunoyama *et al.*, 2002). Overall plastid transcription activity increases early during light-induced plastidial development in order to assure sufficient supply and assembly of the photosynthetic apparatus. However, even in mature leaves transcription of several plastid genes has been shown to be light-induced (Chun *et al.*, 2001; DuBell und Mullet, 1995; Hoffer und Christopher, 1997; Nakamura *et al.*, 2003; Schrubar *et al.*, 1990). Light-induced regulation has also been shown for a number of nuclear encoded genes involved in photosynthesis (Granlund *et al.*, 2009; Terzaghi und Cashmore, 1995; Thompson und White, 1991). Thus, it is tempting to assume that the expression of the nuclear encoded plastid RNA polymerase (NEP) is also regulated by availability and quality of light, as it subscribes not only important plastid housekeeping genes, but also the *rpoB* operon, encoding major subunits of PEP which is accountable for the transcription of most photosynthesis genes in plastids (for reviews see (Hess und Börner, 1999; Liere und Maliga, 2001). In *Arabidopsis*, as in all

dicotyledonous plants analyzed so far, NEP activity is represented by two enzymes, one of which is exclusively targeted to plastids and the other to both plastids and mitochondria (Hedtke *et al.*, 2000). The proteins are encoded by two members of a small nuclear encoded gene family and are named *RpoTp* and *RpoTmp*, respectively. The *RpoT* gene family additionally comprises another member, which is called *RpoTm* and encodes the mitochondrial RNA polymerase (Hedtke *et al.*, 1997; Hedtke *et al.*, 2000; Hedtke *et al.*, 1999).

In the present study I was able to show that steady-state transcript levels of all *RpoT* genes increased when plants were illuminated (3.1.1, Fig, 1; 3.1.2, Fig 2 and Fig. 4). 7-day-old etiolated seedlings that were exposed to white light with an intensity of about 150 µE showed a pronounced increase in transcript accumulation of all *RpoT* genes after six hours of illumination (3.1.1, Fig. 1). Transcript levels were even further increased after twelve hours (3.1.1, Fig. 1). However, no changes in the amount of mRNAs were measured upon exposure of up to one hour (3.1.1, Fig. 1). Hence, the kinetics of *RpoT* gene transcript accumulation resembles those of chloroplast genes like *psbA* and *rbcL* (Hoffer und Christopher, 1997; Klein und Mullet, 1990). Steady-state levels of *psbD* transcripts under control of the blue light responsive promoter (BLRP) in dark grown barley seedlings have been shown to accumulate already after two hours of illumination (Christopher und Mullet, 1994; Gamble und Mullet, 1989; Sexton *et al.*, 1990). In comparison to other light-induced nuclear genes, transcript accumulation of the *RpoT* genes hence progresses relatively slow. One of the most extensively studied nuclear genes whose transcription is regulated by light is *Cab1* (*Lhcb1.3*). It has been shown that its steady-state transcript levels are significantly increased after only one hour upon illumination of etiolated *Arabidopsis* and pea (*Pisum sativum*) seedlings (Gao und Kaufman, 1994; Warpeha *et al.*, 1989).

During the process of de-etiolation the plant undergoes fundamental physiological and molecular changes that are accompanied by differential expression of multiple genes (Jiao *et al.*, 2005; Ma *et al.*, 2001; Ma *et al.*, 2003; Reichler *et al.*, 2001). To exclude that the observed increase in *RpoT* gene transcript levels is merely due to a general increase of the plant's transcription activity when etiolated seedlings undergo photomorphogenesis, the induction experiments were repeated with mature and senescent rosette leaves (3.1.2 and 3.1.3). Accumulation of all *RpoT* genes was still strongly induced by white light in 3-week old and even in 9-week old, senescent leaves (3.1.2, Fig. 2 and Fig. 4). This clearly shows that the effect is not limited to seedlings undergoing photomorphogenesis, but that transcript

accumulation of the *RpoT* genes is as well light induced in mature, green and even senescing rosette leaves.

While the amount of transcripts in 7-day-old seedlings increased continuously even after twelve hours of illumination, the situation was different in mature rosette leaves. In 3-week old, as well as in 9-week old leaves, induction of all three genes was considerably stronger after six hours of illumination as compared to 7-day-old seedlings (3.1.1, Fig, 1; 3.1.2, Fig 2 and Fig. 4). By contrast, accumulation did not increase any further but even decreased strongly upon long-term illumination, indicating the involvement of additional regulative mechanisms. It can be presumed that in mature leaves that are very effectively performing photosynthesis, light has a more pronounced effect on transcription of *RpoT* genes to ensure an optimal adaptation of plastid and mitochondrial functions to increased illumination. High light allows for increased photosynthetic activity and also stimulates respiration and other mitochondrial activities via a complex network between chloroplasts and mitochondria (Noctor *et al.*, 2004; Noguchi und Yoshida, 2008; Raghavendra und Padmasree, 2003; Wertin und Teskey, 2008). Excess light energy also induces the generation of reactive oxygen species (ROS) that impose photo-oxidative damage to the photosynthetic apparatus (Foyer und Noctor, 2003; Hutin *et al.*, 2003; Niyogi, 1999; Roldán-Arjona und Ariza, 2009; Triantaphylides *et al.*, 2008). To avoid profuse production of ROS, plants have developed several mechanisms to dissipate excess light energy (Havaux *et al.*, 2000; Niyogi, 2000; Noguchi und Yoshida, 2008; Shikanai, 2007). It is conceivable that upon strong illumination for a long period plastidial and mitochondrial transcription are additionally downregulated to reduce the rates of photosynthesis and respiration in order to protect the plant from being seriously damaged. *RpoT* gene transcription could thus have considerable influence on the regulation of organellar activity in response to changing light conditions.

There has been a debate recently about the fate of plastid DNA (cpDNA) during leaf maturation and senescence in *Arabidopsis*. Bendich and coworkers stated that cpDNA levels decline during plastid development until most of the chloroplasts contain little or no detectable DNA long before the onset of senescence (Rowan *et al.*, 2004; Rowan *et al.*, 2009). This phenomenon had been reported earlier for several plant species, including barley (Baumgartner *et al.*, 1989), spinach (Scott und Possingham, 1980), rice (Sodmergen *et al.*, 1991), tobacco, maize, pea and *Medicago truncatula* (Oldenburg und Bendich, 2004; Shaver *et al.*, 2008; Shaver *et al.*, 2006). However, two recent studies report a constant amount of cpDNA throughout leaf development in tobacco (Li *et al.*, 2006) and *Arabidopsis* (Zoschke *et al.*, 2007). The fact that both plastid targeted *RpoT* genes are strongly

upregulated by light in mature and even in senescent rosette leaves (3.1.2, Fig 2 and Fig. 4) suggests that transcription of plastid genes is still important during these developmental stages. Thus, the results shown here clearly favor the hypothesis that the plastome of *Arabidopsis* is still present and actively transcribed during early leaf senescence.

It is still unclear why dicotyledonous plants harbor two plastid targeted phage-type RNA polymerases (RpoTp and RpoTmp) and what their respective functions in cpDNA transcription are. According to recent studies using *Arabidopsis*, RpoTp plays a major role in transcription and biogenesis of the chloroplast (Hricová *et al.*, 2006) while RpoTmp is supposed to be mainly active in non-green tissue (Emanuel *et al.*, 2006). However, partial replacement of RpoTp function by RpoTmp was shown in *rpoTp* mutants (Courtois *et al.*, 2007; Swiatecka-Hagenbruch *et al.*, 2008) and light-induced accumulation of several plastid transcripts during early seedling development was shown to be dependent on RpoTmp (Baba *et al.*, 2004). In the present study, accumulation of *RpoTp* transcripts was affected much stronger than *RpoTmp* transcript accumulation in each developmental stage analyzed. Overall relative amount of transcripts was also distinctly higher for RpoTp (3.1.1, Fig, 1; 3.1.2, Fig 2 and Fig. 4). This argues in favor of a pivotal role of the solely plastid targeted enzyme (RpoTp) in the transcription of plastid genes in all analyzed stages of leaf development in *Arabidopsis*. Thus, the data presented here are contrary to a major role of RpoTmp during seedling development and the de-etiolation process as suggested by Baba *et al.* (2004) and rather propose RpoTp to be the key enzyme during light induced plastid transcription. A distinct role of RpoTmp in the transcription of the *rrn* operon during seed imbition (Courtois *et al.*, 2007) is consistent with the data presented here, though.

Plants are not only able to detect and respond to spontaneously changing conditions but they can also adapt to recurring alterations of their environment. The availability of light during day/night cycles is one of the most important periodic changes in nature. As a consequence, many processes in plants are regulated according to this diurnal cycling, i.e. they recur with a frequency of approximately twenty-four hours. If the plant's reactions to these daily oscillations persist under constant conditions and are thus regulated by an independent internal mechanism, they are termed circadian (Barak *et al.*, 2000; Dunlap, 1999; Gardner *et al.*, 2006). Many physiological and molecular processes in higher plants are under the control of such mechanisms (Dowson-Day und Millar, 1999; Jouve *et al.*, 1999; McClung, 2008; Yakir *et al.*, 2007). The endogenous oscillator that coordinates the daily rhythm is called the circadian clock. Many molecular key components of the *Arabidopsis* circadian clock have been identified recently (Alabadi *et al.*, 2001; Ding *et al.*,

2007; McWatters *et al.*, 2007; Millar *et al.*, 1995; Nakamichi *et al.*, 2005; Wang *et al.*, 1997; Wang und Tobin, 1998).

Transcript accumulation of many genes is regulated by the circadian clock (Harmer *et al.*, 2000; Schaffer *et al.*, 2001). Recent studies suggest around one third of the *Arabidopsis* transcriptome to be controlled by circadian rhythms (Michael und McClung, 2003). Photosynthesis is one of the most important biochemical processes performed by plants and it is necessarily dependent on light. Thus, photosynthetical processes have to be adjusted to prevalent light conditions, including day/night cycles. As chloroplasts are major players in photosynthesis, it is not surprising that the expression of many plastid and plastid targeted genes is regulated by the circadian clock (Hassidim *et al.*, 2007; Hwang *et al.*, 1996; Matsuo *et al.*, 2006; Michael und McClung, 2003; Morikawa *et al.*, 1999; Nakahira *et al.*, 1998; Schaffer *et al.*, 2001). One way to influence plastid transcription is to regulate the expression of NEP, so it was tempting to investigate if transcription of *RpoTp* and/or *RpoTmp* is under control of the circadian clock.

The analyses revealed that the amount of transcripts of all *RpoT* genes oscillates with a very weak diurnal rhythm which is persistent under constant conditions (3.1.3, Fig. 5A). Transcript abundance was highest in early morning und reached a minimum in the afternoon, before increasing again towards the night. Compared to transcript accumulation patterns of genes known to be under strong influence of the circadian clock, such as *CCA1* (3.1.3, Fig. 5B; (Lu *et al.*, 2009; Mizoguchi *et al.*, 2002; Wang *et al.*, 1997; Wang und Tobin, 1998) the oscillation is marginal, though. According to the data achieved here, the circadian clock presumably has no distinct influence on *RpoT* transcript accumulation and gene expression. The only small changes occurring during the diurnal oscillations provide further evidence for a distinct light induction of *RpoT* gene transcription by excluding an influence of circadian effects on the results shown in chapters 3.1.1 and 3.1.2. However, it cannot be ruled out that there is a very slight circadian regulation of *RpoT* transcript accumulation, which could affect transcription of NEP transcribed genes. In favor of this, it was found that *rpoB* transcripts also accumulated with a marginal circadian rhythm, peaking a few hours later than *RpoTp* and *RpoTmp* transcripts (data not shown). It is possible that slightly higher amounts of NEP transcripts lead to an increased transcription rate in plastids and thus to the accumulation of transcripts of NEP-transcribed genes like *rpoB*. It has been shown previously that the amount of transcripts of plastid genes known to be transcribed by NEP, including *rpoB*, closely followed the pattern of *RpoTp* transcript accumulation in a developmental leaf gradient of barley (Emanuel *et al.*, 2004).

Plants possess a variety of different photoreceptors (Chen *et al.*, 2004; Jiao *et al.*, 2007), including the red/far-red light absorbing phytochromes (Franklin und Whitelam, 2004; Smith, 2000) and the UV-A/blue light absorbing cryptochromes (Li und Yang, 2007; Lin und Shalitin, 2003), phototropins (Briggs und Christie, 2002; Christie, 2007; Inoue *et al.*, 2008) and members of the Zeitlupe/FKF1/LKP2 family (Briggs, 2007; Imaizumi *et al.*, 2003; Kim *et al.*, 2007; Ulm und Nagy, 2005). Supposedly, there are even more, yet unknown receptors that may absorb in green, UV-B or any other light quality (Dhingra *et al.*, 2006; Ioki *et al.*, 2008; Talbott *et al.*, 2003). Obviously, regulation of light responses mediated by this multitude of enzymes involves complex pathways, most of which have not been thoroughly clarified as to now. In the present study it was shown that transcription of the *RpoT* gene family in *Arabidopsis* is strongly induced by light. To understand more about the underlying molecular events it is crucial to understand which photoreceptors play a role in the signaling cascade. To this purpose, wildtype seedlings were illuminated with monochromatic light of different wavelengths and additionally, photoreceptor knockout mutants were analyzed.

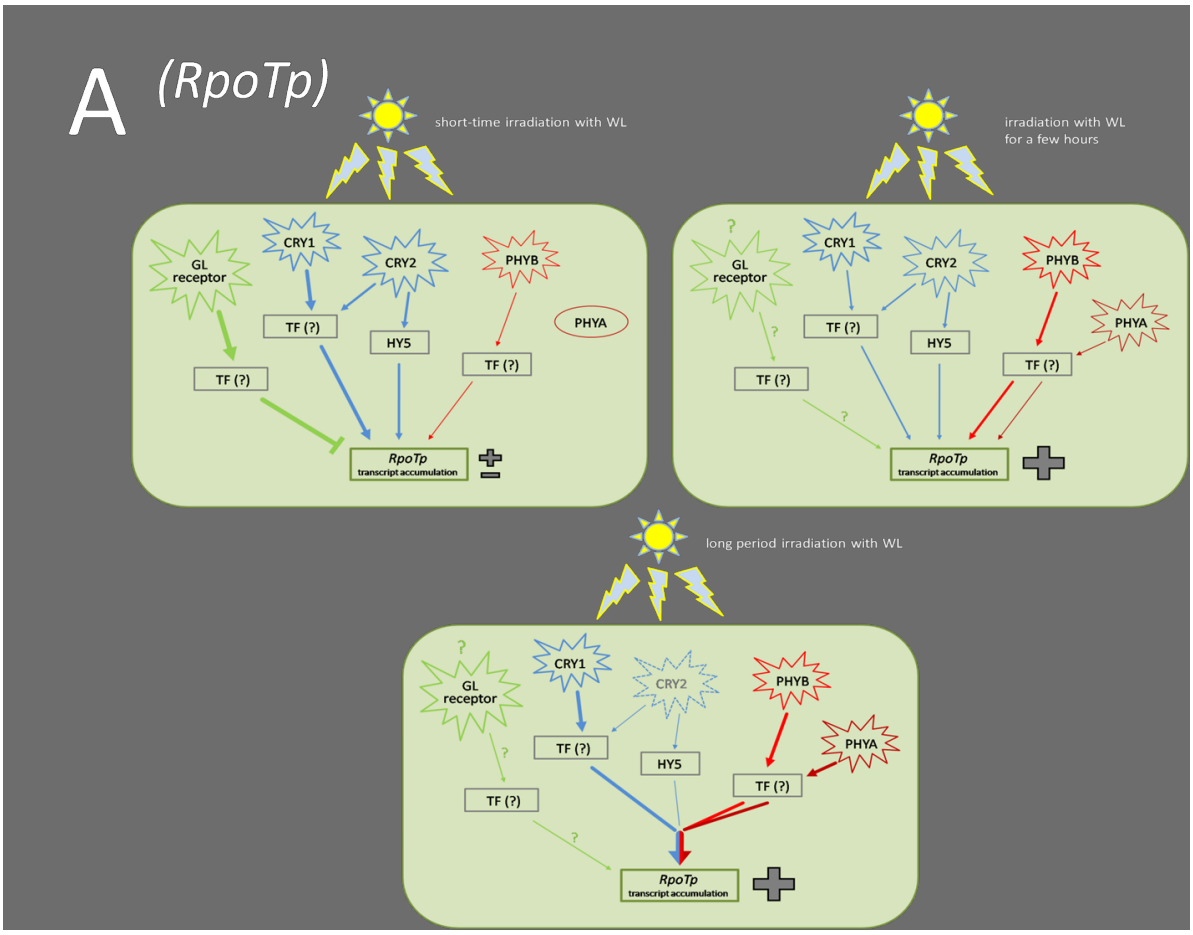
The results presented in chapters 3.1.4 and 3.1.5 indeed suggest a very complex network of different photoreceptors acting in different light qualities to ensure a correct transcription of the *RpoT* genes in response to light. Not only are multiple photoreceptors involved in the regulation of different *RpoT* genes, but also their participation in the network differs between plastid, mitochondrial and dually targeted enzyme.

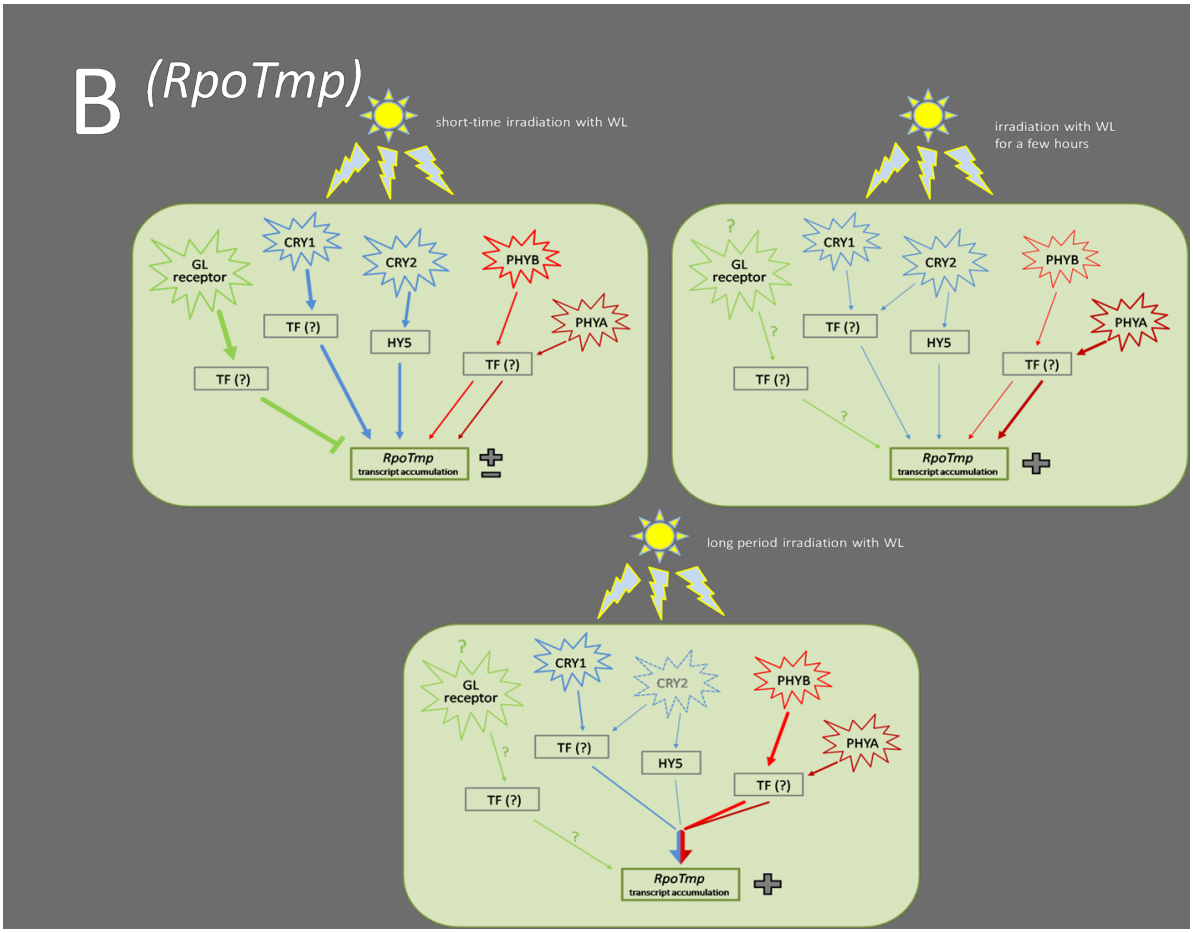
Taken together, the results obtained from the analyses of transcript accumulation in 7-day-old seedlings exposed to red, blue and green light and the use of different phytochrome and cryptochrome knockout mutants suggest that influence and importance of different photoreceptors changes in the course of illumination. Long-term irradiation involves entirely different sets of photoreceptors as does to short-term illumination. Furthermore the proportion of different light qualities plays an important role for the regulation of *RpoT* gene transcript accumulation. Generally, light does not lead to any increase in the amount of transcripts until several hours of illumination.

Most interestingly exposure to monochromatic green light leads to a strong decrease in the amount of all *RpoT* transcripts after only one hour (3.1.4 Fig. 8). Later, induction takes place similar as in white light. This suggests a very important and unique role of green light in the first minutes to hours of light induced *RpoT* gene expression. Green light was recently shown to have pronounced effects on early developmental processes (Dhingra *et al.*, 2006; Folta, 2004; Folta und Maruhnich, 2007). It has been proposed that typical blue light

responses, such as stomatal opening are counteracted and even reversed by green light (Eisinger *et al.*, 2003; Frechilla *et al.*, 2000; Talbott *et al.*, 2006). Furthermore it was shown that green light strongly effects transcription of plastid genes. When etiolated *Arabidopsis* seedlings were treated with a short, dim pulse of green light, multiple plastid-encoded transcripts were found to decrease in abundance within thirty minutes (Dhingra *et al.*, 2006).

The data presented here are consistent with these findings. Exposure of etiolated seedlings to green light quickly depletes the accumulation of *RpoT* genes (3.1.4 Fig. 8). As this involves a downregulation of NEP transcripts it could be an explanation for decreasing plastid transcript abundance. Dhingra *et al.* (2006) concluded that the green light effect on plastid transcripts is most likely achieved by a new type of photoreceptor rather than by known photoreceptors, because it persisted in all photosensory mutant lines tested (*phyA*, *phyB*, *hy1*, *cry1cry2*, *phot1phot2* and *npq1*). Additionally, blue and red light as well as a mixture of both wavelengths did not lead to a decrease in the amount of transcripts (Dhingra *et al.*, 2006). The present study similarly shows that most probably a photoreceptor other than phytochrome or cryptochrome is responsible for the green light effect on *RpoT* gene transcript abundance (Fig. 1).


A (RpoTp)
short-time irradiation with WL
irradiation with WL for a few hours
long period irradiation with WL
GL receptor
CRY1
CRY2
PHYB
PHYA
TF (?)
HY5
RpoTp transcript accumulation


B (RpoTmp)
short-time irradiation with WL
irradiation with WL
for a few hours
long period irradiation with WL
GL receptor
CRY1
CRY2
PHYB
PHYA
TF (?)
HY5
RpoTmp
transcript accumulation

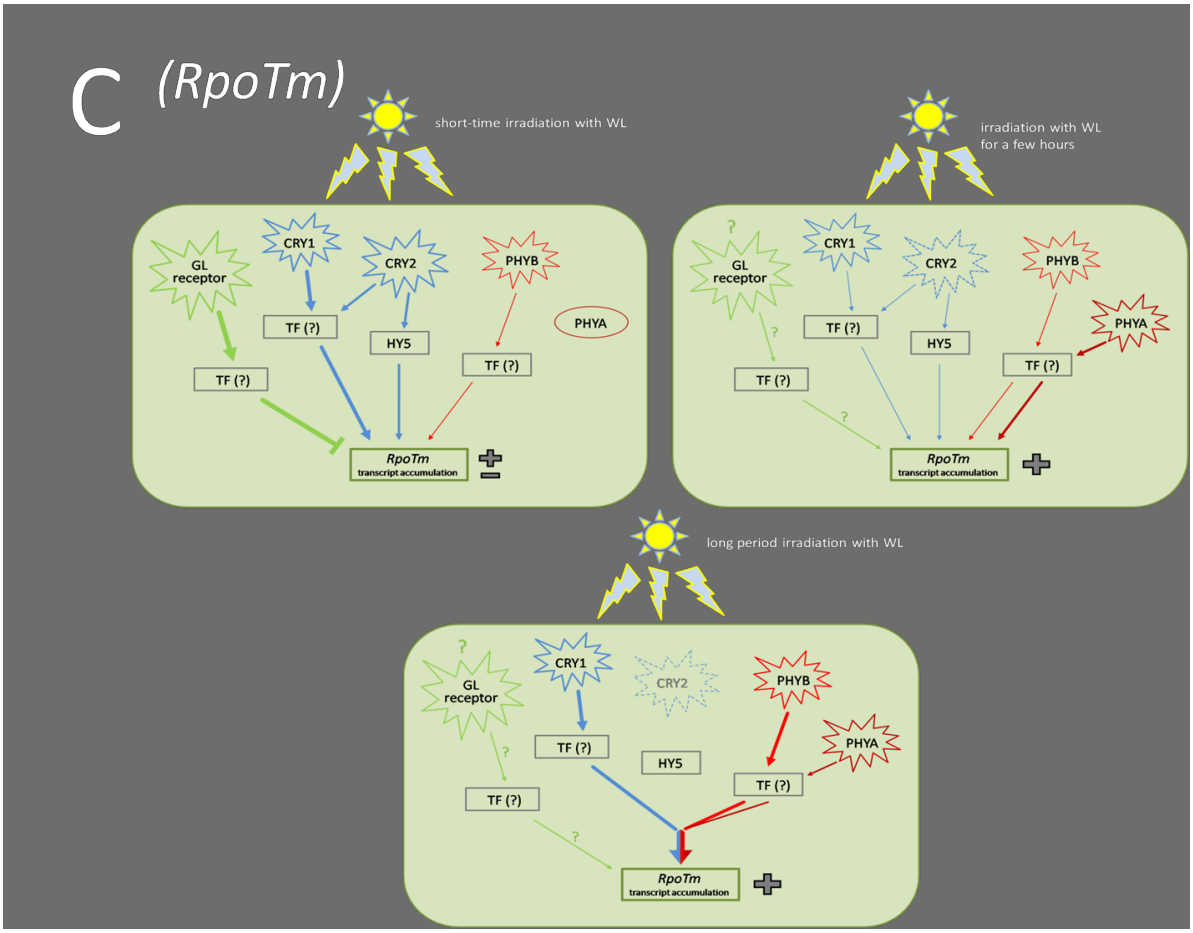


Figure 1: Model for the involvement of different photoreceptors in the accumulation of *RpoT* gene transcripts in etiolated *Arabidopsis* seedlings upon illumination with white light.
Based on the data obtained in the different experiments (see chapter 3.1) a model was developed that shows the involvement of PHYA, PHYB, CRY1, CRY2 and an unknown photoreceptor ("GL receptor") in light-induced transcript accumulation of the *RpoT* genes. An arrow indicates a positive effect on transcript accumulation, a line with a blunt end indicates an inhibitory effect. Thickness of the lines corresponds to the importance of the respective receptor. Short-time and long period irradiation means illumination for around one hour and twelve hours, respectively. Quickly upon irradiation of etiolated seedlings, green light, most probably via an unknown photoreceptor, has an inhibitory effect on transcript accumulation of all three *RpoT* genes. CRY1 seems to be the major photoreceptor regulating a positive effect on transcript accumulation at that time, while the roles of CRY2 and PHYB are minor. PHYA at that time only seems to play a role in the accumulation of *RpoTmp* (B). After several hours of irradiation, the inhibitory green light effect disappears and the impact of phytochromes becomes stronger. PHYA now plays a major role in the light induction of *RpoTmp* (B) and *RpoTm* (C), while PHYB seems to be an important receptor for *RpoTp* light induction (A). When plants are illuminated for a long period (~12 h), transcript accumulation of all *RpoT* genes requires a concerted action of CRY1, PHYA and PHYB, while CYR2 is suggested to be mostly degraded by that time.

The unknown receptor (termed "GL receptor" in Fig. 1) supposedly promotes a decrease in *RpoT* transcript levels by either negatively influencing transcription or destabilizing transcripts. The fact that transcript abundance quickly decreases upon illumination rather

than remaining constant favors the latter scenario, because by preventing transcription alone, a decrease in steady-state levels would hardly be detectable that fast.

The results also show that the negative effect of green light is neutralized after an illumination for several hours (3.1.4 Fig. 8). It seems obvious that the role of cryptochromes and phytochromes in the light induction process becomes more important the longer the seedlings are illuminated. After four hours of illumination, transcript accumulation in green light progresses similar as in white light (3.1.4 Fig. 8). Hence, most probably phytochromes and/or cryptochromes, which are known to be able to absorb in green light (Banerjee *et al.*, 2007; Bouly *et al.*, 2007; Folta und Maruhnich, 2007; Mandoli und Briggs, 1981; Shinomura *et al.*, 1996), are promoting the slow induction after several hours in green as well as in blue and white light. It is thus suggested that the effect of green light most probably promoted by a yet unknown photoreceptor is of importance only in the first few minutes to hours of illumination provided a large proportion of green light. This hypothesis is supported by data from Dhingra *et al.* (2006), showing that abundance of some plastid transcripts decreases already after fifteen minutes, reaches a minimum after one hour and only slightly tends to increase after two hours in green light (Dhingra *et al.*, 2006).

In an evolutionary context this regulative mechanism allows the plant to react to situations where wavebands are not favorable for photosynthesis, such as growing under a canopy or in dim light. In this case a separate genetic program, described as hyperskotomorphogenic by Dhingra *et al.* (2006), assures preservation of resources by the plastids and allows the plant to grow and reach better light conditions. As the seedlings analyzed here are etiolated, reduced plastid transcription will effectively inhibit proper development of chloroplasts and loading of the thylakoids with photosynthetic protein complexes to save considerable amounts of energy. Even if transcription is induced through other photoreceptors upon long-term exposure to such unfavorable conditions, the total amount of *RpoT* gene transcripts will, due to the strong early decrease, stay considerably below the level reached in blue or white light. This will eventually lead to less NEP and thus overall plastid transcription will be lower. Further work has to be done to identify the receptor(s) responsible for this response.

In the present study, *hy5* mutants were additionally analyzed, because HY5 is known to be an important transcription factor acting downstream of phytochromes, cryptochromes and other photoreceptors (Ang *et al.*, 1998; Koornneef *et al.*, 1980; Oyama *et al.*, 1997; Ulm *et al.*, 2004). It has been shown that more than 60% of early PHYA- or PHYB-induced genes are targets of HY5 (Lee *et al.*, 2007). Thus, HY5 seems to be a high hierarchical

regulator in plant photomorphogenesis (Lee *et al.*, 2007). The present study, however, shows that the role of HY5 in the network regulating light-induced *RpoT* gene expression is only minor. Knockout of the *HY5* gene did not have major effects on light-induced accumulation (3.1.5, Fig. 15). However, *hy5* mutants showed a slight decrease of transcripts after one hour of illumination, similar to what had been observed in cryptochrome knockout-mutants (3.1.5, Figs. 10-12). This suggests HY5 to play a role downstream of cryptochromes early during de-etiolation. The *hy5* mutant does not show severe effects upon long-term illumination, similar to the *cry2* mutant. It is therefore supposed that HY5 acts downstream of CRY2 rather than CRY1, because the latter obviously still is of great importance after long periods of irradiation (Fig. 1). Interestingly, the data gives no evidence whatsoever for an involvement of HY5 in phytochrome mediated effects on light-induced *RpoT* gene transcript accumulation.

In summary, together with recent results (Dhingra *et al.*, 2006; Folta, 2004; Folta und Maruhnich, 2007) the data presented here suggest that regulation of *RpoT* gene, and thus NEP, transcript accumulation by green light has major impact on plastid transcription and seedling development. Dhingra *et al.* (2006) conducted time course analyses that showed a green-light induced down-regulation of plastid transcripts after only fifteen minutes. They concluded from their experiments that due to the rapid reaction, NEP-based transcription is likely not affected by green light, but that the resulting transcripts are destabilized (Dhingra *et al.*, 2006). This is in agreement with our data, which similarly showed a very quick decrease of steady-state transcript levels that can hardly be achieved solely by prevention of transcription.

The model above shows that, according to the data achieved, the rapid negative effect of green light that is visible after only one hour of illumination, is, under optimal light conditions, neutralized by the inducing effect of blue light mediated mainly through cryptochromes 1 and 2 (Fig. 1 A-C, top left). If either *CRY1* or *CRY2* are knocked out, the amount of all *RpoT* transcripts decreased significantly after only one hour of illumination with white light, very similar to the effect observed in wildtype plants under green light (3.1.4, Fig. 8 and 3.1.5, Figs. 10-12). Phytochromes do not seem to play an important role in the early regulation of *RpoTp* and *RpoTm* transcript accumulation, though. Loss of *PHYA* did not have any effect at all (3.1.5, Fig. 12) and plants lacking *PHYB* only showed a marginal decrease in transcript levels (3.1.5, Fig. 13), suggesting a positive, albeit relatively small induction via a pathway involving phytochrome B. However, in the case of *RpoTmp* both phytochromes seem to be positively involved in transcript accumulation to a larger

degree (3.1.5, Figs. 13-14 B). Here, knockout of either *PHYA* or *PHYB* led to a decreased amount of transcripts shortly after irradiation with white light.

In summary, very early changes in *RpoTp* and *RpoTm* transcript accumulation in response to light are supposedly regulated via similar receptors and pathways, while regulation of *RpoTmp* transcript levels seems to involve slightly different mechanisms. Both *RpoTm* and *RpoTp* transcript accumulations are suggested to be strongly induced via cryptochromes and slightly via phytochrome B, while transcript stability is most likely decreased effectively via a mechanism involving a yet unidentified photoreceptor absorbing in the green waveband. In broadband white light, the two opposing mechanisms lead to a constant amount of transcripts during the first hours of illumination. *RpoTmp* transcript accumulation seems to be regulated very similar, but additionally involves phytochrome A as photoreceptor in a positively regulating pathway.

After several hours of illumination the influence of cryptochromes becomes less important and involvement of phytochromes increases for all *RpoT* transcripts (Fig. 1 A-C, top right). Surprisingly, PHYA, which is known to be degraded rapidly in light (Clough und Vierstra, 1997), now seems to play a role in the accumulation of *RpoTp* and *RpoTm* transcripts. Additionally, it still seems to be the major photoreceptor for the regulation of *RpoTmp* transcript accumulation. Altogether, at that stage all photoreceptors analyzed are to some extent involved in light induced *RpoT* gene transcript accumulation. Upon further illumination this phenomenon becomes even more important. After twelve hours of illumination knockout of each of the analyzed photoreceptor genes leads to a strong decrease in the amount of transcripts (Fig. 1 A-C, bottom), while in white light accumulation actually increases (3.1.1, Fig.1). This suggests that a complex network of multiple photoreceptors is needed to regulate *RpoT* gene expression at this stage. However, loss of CRY2 can be almost completely compensated. CRY2 is thought to be mainly active in low-light conditions and during the early development of seedlings, because its expression is strongly downregulated by blue light (Ahmad *et al.*, 1998; Lin *et al.*, 1998). This would explain why the importance of CRY2 in light induction processes diminishes upon long-period illumination.

Although the phytochromes obviously play a major role in the regulation of *RpoT* gene expression upon longer illumination, red light alone was not able to invoke any further transcript accumulation (3.1.4, Fig.7). This suggests that the phytochrome response in this case is mainly triggered by blue light, which is readily absorbed by these receptors (Goto *et al.*, 1993; Shinomura *et al.*, 1996).

Taken together, the present study shows that light induction of *RpoT* gene transcript accumulation involves the interaction of a variety of different photoreceptors and pathways. In recent years it has become more and more obvious that light induced processes like photomorphogenesis are often regulated on multiple levels and through many different interconnected pathways. Thus, complex networks of many photoreceptors seem to be the rule rather than an exception (Eisinger *et al.*, 2003). Functional interactions have been shown especially for cryptochromes and phytochromes during de-etiolation (Ahmad und Cashmore, 1997; Ahmad *et al.*, 1998; Guo *et al.*, 1998; Más *et al.*, 2000; Mockler *et al.*, 1999; Neff und Chory, 1998; Thum *et al.*, 2001).

Against this background it is not surprising that light-induced regulation of *RpoT* gene transcription is not only dependent on one class of receptors, but utilizes the whole spectrum of phytochromes, cryptochromes and most probably additional photoreceptors. Further examinations involving mutants lacking other known or putative photoreceptors and transcription factors, carried out under different light regimes could further clarify the pathways of light-regulated *RpoT* gene expression.

To further address the question which photoreceptors play a role in light induced expression of the *RpoT* genes in *Arabidopsis* and to elucidate the network of downstream pathways it is important to conduct continuative experiments. Analyses of transcript accumulation in the different photoreceptor mutants upon illumination with red, blue and green light or combinations of different wavelengths will probably shed more light on this issue. Additionally, the effect of different light qualities on transcript levels of plastidial and mitochondrial genes transcribed by nuclear encoded RNA polymerases will be very interesting. To further investigate the relevance of the circadian rhythm on the expression of NEP-transcribed genes, it will be worth analyzing diurnal accumulation patterns of plastid genes. Moreover, run-on analyses could clarify the impact of transcript stability and *de novo* synthesis in these processes.

4.2 Organellar gene copy numbers and transcript levels in chlorophyll-deficient tissue

4.2.1 Analysis of *RpoT* gene expression during leaf development in *Arabidopsis thaliana*

Despite the very complex regulative mechanism shown above, light is most probably not the only stimulus *RpoT* gene expression reacts to. In previous studies it has been shown that

RpoT transcripts accumulate differentially depending on tissue and developmental state (Emanuel *et al.*, 2006). Furthermore it has been shown that transcriptional activity of plastid genes is reduced profoundly in older rosette leaves compared to cotyledons and young leaves (Zoschke *et al.*, 2007). The plastid encoded RNA polymerase, PEP, was supposed to play a major role in the transcription of plastid genes in older leaves, whereas NEP activity seemed to decline (Zoschke *et al.*, 2007).

To further analyze the relevance of *RpoTp* and *RpoTmp*, but also of *RpoTm* expression during leaf development in *Arabidopsis*, transcript accumulation was monitored in leaf samples ranging from 2-day-old cotyledons to 37-day-old, senescent rosette leaves. The observed progression of *RpoTp* transcript accumulation supports the results of Zoschke *et al.* (2007) and Emanuel *et al.* (2005). In very young cotyledons, steady-state levels were relatively low and they increased drastically towards a maximum in 13-day-old rosette leaves (3.1.6, Fig. 16C). From there on, the amount of mRNA rapidly declined and was found to be even lower in senescent leaves than in young cotyledons. *RpoTmp* transcripts on the other hand were found to be most abundant in cotyledons and senescent leaves, albeit overall abundance was lower than that of *RpoTp* transcripts, except for the oldest analyzed leaves (3.1.6, Fig. 16B). Interestingly, the progression of *RpoTmp* transcript accumulation is directly opposed to that of *RpoTp* transcripts, with a minimum in mature, green leaves. This supports the idea that RpoTp plays a major role in green tissue, whereas RpoTmp fulfills a different, albeit probably slightly redundant role, for example very early in seedling development, in plastids of non-green tissue and supposedly also in mitochondria (Courtois *et al.*, 2007; Demarsy *et al.*, 2006; Emanuel *et al.*, 2006; Kühn *et al.*, 2007). Transcripts of *RpoTm*, encoding the mitochondrial RNA polymerase, show a very similar accumulation pattern as those of *RpoTmp* (3.1.6, Fig. 16A) suggesting a similar expression of the corresponding enzymes. This would probably be a hint towards a mitochondrial function of RpoTmp. Anyhow, the data clearly show a profoundly different expression of *RpoTp* on one and *RpoTm* and *RpoTmp* on the other side, suggesting distinct roles for both NEP-candidates in *Arabidopsis* leaves. Furthermore, a prevalent role for RpoTp in mature, green tissue and reduced importance in older leaves was shown. The data thus support the idea of PEP becoming more important for the transcription of plastid genes later in leaf development (Zoschke *et al.*, 2007).

RpoTm transcripts are expected to be most abundant in tissue with high mitochondrial activity. This is certainly true for very young cotyledons, where the amount of *RpoTm* transcripts was indeed found to be at a maximum (3.1.6, Fig. 16A) before decreasing in

rosette leaves. Interestingly, steady-state levels increase again in older leaves with a second maximum in early senescent leaves around day 27, suggesting enhanced mitochondrial activity at this point of development.

4.2.2 Light-induced transcript accumulation of plastid genes in green and chlorophyll-deficient *Arabidopsis* seedlings

The transcriptional machinery of plant organelles is surprisingly complex. Besides the plastid encoded, eubacterial-type RNA polymerase (PEP) there are additional polymerases that are encoded in the nucleus and resemble enzymes of the phage-type (for a review, see (Liere und Börner, 2007). One of them (RpoTm) is exclusively targeted to mitochondria where transcription is solely accomplished by nuclear encoded RNA polymerases. The second protein (RpoTp) is imported into plastids and in dicotyledonous plants the situation is even more complex in that a third polymerase (RpoTmp) exists which is dually targeted to both types of organelles (Hedtke *et al.*, 1997; Hedtke *et al.*, 2000). Hence, in *Arabidopsis*, as in all dicots analyzed so far, three polymerases are transcribing the plastome. Not much is known until now about the division of labor between these enzymes, and especially the function of RpoTmp remains largely unclear. There have been several studies in recent years proposing models for a concerted action of the different polymerases and for their distinct roles in different tissue, organs and during development (Baba *et al.*, 2004; Courtois *et al.*, 2007; Demarsy *et al.*, 2006; Emanuel *et al.*, 2006; Swiatecka-Hagenbruch *et al.*, 2008). It has been proposed that RpoTmp is active very early in seedling development (Courtois *et al.*, 2007) and that it transcribes a distinct subset of plastid genes (Courtois *et al.*, 2007; Swiatecka-Hagenbruch *et al.*, 2008). A gene-specific transcription activity for RpoTmp in mitochondria of *Arabidopsis* has very recently also been described (Kühn *et al.*, 2009).

To get further insight into the function of plastid RNA polymerases, transcript accumulation of two plastid genes, *clpP* and *rrn16*, was analyzed by quantitative real-time PCR. These genes were chosen, because they both possess two different promoter recognition sites. While *clpP* is supposed to be transcribed from a strong type-II NEP-promoter (P*clpP*-58) and a relatively weak PEP promoter (P*clpP*-115), *rrn16* possesses a strong PEP (P*rrn16*-112) and the weak NEP-promoter P*rrn16*-139 (Pc-type; (Sriraman *et al.*, 1998; Sriraman *et al.*, 1998; Vera und Sugiura, 1995). Transcript steady-state levels were measured in 7-day-old etiolated seedlings that were exposed to white light for up to

twenty-four hours (see 3.1.1). Additionally, seedlings were grown on medium containing spectinomycin, an antibiotic that inhibits plastid translation and leads to seedlings with white leaves and photosynthetically inactive plastid lacking PEP activity (Hess *et al.*, 1994; Moazed und Noller, 1987; Svab *et al.*, 1990; Wallace *et al.*, 1974; Zubko und Day, 1998).

In the case of *rrn16*, transcript levels increased slightly after just one hour, then decreased again and showed a broad maximum after six hours of illumination (3.2.1, Fig. 19). This corresponds well to the data achieved for *RpoTp* and *RpoTmp* transcript levels. NEP transcript accumulation supposedly increases before six hours of illumination (3.1.1, Fig. 1 and 3.1.4). Thus, from this time point more NEP can transcribe more PEP subunits and with a small delay, more *rrn16* transcripts can be produced by PEP. In white seedlings that lack PEP, transcript levels of *rrn16* were generally much lower and showed only little light induction after twelve hours, supporting the idea that this gene is transcribed mainly by PEP. Interestingly, in the dark, transcript levels were even higher than in green seedlings. This could be explained by a compensatory effect of RpoTmp which is supposed to strongly transcribe from the *rrn16* Pc-promoter very early during seedling development (Courtois *et al.*, 2007) while it is less active later. If this is the case, a retrograde signal must be involved, informing the nucleus about the lack of PEP and/or photosynthesis, ultimately leading to enhanced activity of NEP in form of RpoTmp.

The data for *clpP* transcript accumulation gave a more complex picture (3.2.1, Fig. 18). In green seedlings, a strong increase in transcript levels was found after six hours, again being consistent with data for NEP transcript accumulation. However, after twelve hours, the amount of *clpP* transcripts was reduced markedly, while NEP transcripts still accumulated at this time point (3.1.1, Fig. 1). Most probably *clpP* transcript accumulation is regulated post-transcriptionally via transcript stability upon long-term illumination. Surprisingly, transcript levels were again higher in white plants lacking PEP activity. Furthermore, transcripts accumulated strongly after one hour of illumination in white seedlings, before the amount decreased again. Except for six hours after illumination, transcript levels were generally higher in white than in green tissue. This suggests that P*clpP*-115, which was supposed to be a PEP-promoter, is either very weak or a different kind of promoter than thought previously, because the lack of PEP does not lead to any decrease in the amount of transcripts, but rather an increase. It was recently shown that P*clpP*-115 behaved untypically for a PEP-promoter and that it might actually be a NEP-promoter instead. The promoter region of *clpP* contains -10/-35 elements as well as a possible YRTA motif, thus it is still unclear which polymerases transcribe the gene

(Swiatecka-Hagenbruch, 2008). The data presented here strongly support this finding. The *clpP* gene was also reported to probably be transcribed by RpoTmp (Swiatecka-Hagenbruch *et al.*, 2008), which could explain the strong accumulation of transcripts in darkness. The lack of PEP might lead to a higher rate of transcription by RpoTmp in the dark, an effect that has also been found for *rrn16* transcript accumulation (see above). However, the strong and early induction after only one hour in light has not been observed in the case of *rrn16* and thus supposedly results from enhanced RpoTp activity or altered transcript stability as a compensatory effect of the spectinomycin treatment.

4.2.3 Transcript levels of mitochondrial genes in green and chlorophyll-deficient *Arabidopsis* seedlings

Phage-type RNA polymerases not only transcribe genes in plastids, but also in mitochondria. In dicotyledonous plants, two nuclear encoded enzymes are targeted to mitochondria, RpoTm and the dually targeted RpoTmp (Hedtke *et al.*, 2000; Hedtke *et al.*, 1999). As in the case of plastids, a distinct role for RpoTmp in chondrome transcription has not yet been shown. In a recent study, Emanuel *et al.* (2006) utilized GUS-Assays, *in situ* hybridization and quantitative real-time PCR experiments to show that the expression patterns of *RpoTm* and *RpoTmp* overlapped, while *RpoTp* showed completely different expression. They concluded that RpoTmp actively transcribes genes in mitochondria, although probably recognizing different types of promoters (Emanuel *et al.*, 2006). Another very recent study supports a distinct function of RpoTmp in *Arabidopsis* mitochondria by providing evidence for a gene-specific alteration of transcription and transcript accumulation in *rpoTmp* mutants (Kühn *et al.*, 2009).

In previous studies using the barley *albostrians* mutant it has been shown that expression of mitochondrial genes is influenced by the status of plastids (Emanuel *et al.*, 2004; Hedtke *et al.*, 1999). The *albostrians* mutant has both normal green leaves and white leaves, which lack ribosomes and are photosynthetically inactive (Hess *et al.*, 1993; Hess *et al.*, 1994; Hess *et al.*, 1992). The authors showed enhanced transcript levels of mitochondrial as well as *RpoT* genes in the white leaves (Emanuel *et al.*, 2004; Hedtke *et al.*, 1999). Increased accumulation of *RpoT* gene transcripts was also observed recently in *Arabidopsis* seedlings that had been grown on spectinomycin-containing medium (Swiatecka-Hagenbruch, 2008).

In the present study it could be shown that transcript levels of four representative mitochondrial genes (*atp1*, *cox1*, *rps4* and *nad6*) were significantly increased in 10- and 20-

day-old white, spectinomycin-treated compared to normal, green *Arabidopsis* plants (3.2.2, Fig. 20). *RpoTm* and *RpoTmp* transcript levels were previously found to be five- and twofold increased, respectively, in 21-day-old, chlorophyll-deficient seedlings of *Arabidopsis* (Swiatecka-Hagenbruch, 2008). It can therefore be assumed that increased expression of RpoTm and RpoTmp, representing the mitochondrial transcription machinery (Swiatecka-Hagenbruch *et al.*, 2008) concomitantly leads to higher accumulation of mitochondrial gene transcripts in white seedlings. A reason for this complex reaction, involving sophisticated signaling mechanisms between plastids, mitochondria and the nucleus could be the lack of photosynthetic energy that forces the cell to enhance mitochondrial respiration in order to cover the cell's energy demands. Plastid status and photosynthetic activity thus seem to have direct and distinctive influence on nuclear and mitochondrial transcription.

4.2.4 Mitochondrial gene copy numbers in green and chlorophyll-deficient *Arabidopsis* seedlings

Hedtke *et al.* (1999) found mitochondrial transcripts in white leaves of the barley *albostrians* mutant to be increased 1.5- to twofold compared to green leaves (Hedtke *et al.*, 1999). This is in good agreement with data from chlorophyll-deficient *Arabidopsis* plants achieved in the present study, where 1.2- to threefold changes were observed (3.2.2, Fig. 20). In barley, the authors additionally found elevated levels of mitochondrial DNA, with gene copy number increased 2.5- to threefold in white leaves (Hedtke *et al.*, 1999). They concluded that the lack of differentiated plastids in otherwise normal leaves most probably leads to a higher replication rate of the mitochondrial genome which then results in enhanced levels of mitochondrial gene transcripts. In order to investigate if a comparable coherence also exists in *Arabidopsis*, gene copy numbers of the four previously analyzed mitochondrial genes were measured by quantitative real-time PCR in green and white plants after 10 and 20 days. Nuclear endopolyploidization levels were determined, in order to be able to normalize the data correctly. Interestingly, white seedlings showed a slightly higher amount of nuclear genome copies than green leaves (3.2.3, Fig. 21). Analysis of *atp1*, *rps4*, *cox1* and *nad6* gene copies revealed considerably increased numbers in chlorophyll-deficient compared to green plants (3.2.2, Fig. 22). In 10-day-old seedlings, numbers were 3.4 to 4.3 times higher and after twenty days they were 2.2 to 2.5 times higher, which again corresponds well with the data obtained previously from the *albostrians* mutant (Hedtke *et*

al., 1999). The elevated numbers of mitochondrial gene copies could either result from an increased number of mitochondria or from increased replication of mtDNA per mitochondrion. The latter was suggested to be the case in white leaves of *albostrians* mutants, where no increase in the number of discrete mitochondria was detected in white compared to green leaves (Hedtke *et al.*, 1999). By contrast, two recent studies found elevated number of mitochondria in albino mutants of *Phaseolus vulgaris* (Soares *et al.*, 2005) and *Secale cereale* (Ballesteros *et al.*, 2009) that were also attributed to a compensatory effect due to the lack of photosynthesis. However, the different scenarios are two ways to achieve an equal aim, namely an increase in mitochondrial DNA.

The data presented here show that in *Arabidopsis*, lack of differentiated chloroplasts and photosynthesis leads to both increased mitochondrial gene copy numbers and enhanced accumulation of *RpoTm* and *RpoTmp* transcripts. It is conceivable that both effects play a role in the enhanced transcription of mitochondrial genes in those plants. The data presented here gives further proof for the extremely complex coordination of organellar and nuclear gene expression, especially in photosynthetic organisms (recently reviewed in (Woodson und Chory, 2008).

4.2.5 Mitochondrial gene copy numbers during leaf development in *Arabidopsis thaliana*

If mitochondrial gene copy numbers and transcript levels are influenced by the developmental state of plastids and the photosynthetic apparatus, it is tempting to speculate that there might also be changes during leaf development. To this end, mitochondrial gene copy numbers were determined in leaves of different ages, ranging from 5-day-old cotyledons to 36-day-old, senescent rosette leaves (3.3.1, Fig. 23). It has been shown before that copy numbers of the plastome do not change significantly between these different leaf age stages (Zoschke *et al.*, 2007). However, copy numbers of the four analyzed representative mitochondrial genes were found to be relatively high in young cotyledons, very low in stem leaves and additionally showed a striking increase in older rosette leaves (3.3.1, Fig. 23). This significant increase was found for all analyzed genes between day 27 and day 31 (Ros50/4 to Ros50/6) of rosette leaves from 50-day-old plants. Around day 28 (Ros50/5), the leaves also showed first signs of early senescence (see Fig. 2). Thus, a correlation between senescence processes and the increase in mitochondrial gene copy

numbers could be assumed. When leaves started to bleach (Ros50/7 and Ros50/8), the number of gene copies decreased drastically again.

Figure 2: Appearances of *Arabidopsis* rosette leaves of different ages.
Denomination of the different leaf age stages is defined in Zoschke *et al.* (2007).

Senescence, the final developmental phase of leaves is a complex and highly organized process, which is very important for the viability of the whole plant. During developmental or age-induced senescence, nutrients are mobilized from the dying leaves to support growth and development of other organs, such as young leaves, flowers or developing seeds (Feller und Fischer, 1994; Himelblau und Amasino, 2001; Hörtensteiner und Feller, 2002; Lim *et al.*, 2007; Masclaux *et al.*, 2000; Quirino *et al.*, 2000). Leaf senescence is a genetically programmed process that involves many changes not only in gene expression, but also in metabolic processes (Balazadeh *et al.*, 2008; Buchanan-Wollaston *et al.*, 2003; Buchanan-Wollaston *et al.*, 2005; Gepstein *et al.*, 2003; Guo *et al.*, 2004; Lin und Wu, 2004). Although the mechanisms leading to the onset of senescence and finally to programmed cell death (PCD), which often stands at the end of the senescence program (Thomas *et al.*, 2003; van Doorn und Woltering, 2004) are not fully understood, it is obvious that mitochondria play an important role in both processes (Andersson *et al.*, 2004; Bhalerao *et al.*, 2003; Blackstone und Kirkwood, 2003; Keskitalo *et al.*, 2005; Krause und Durner, 2004; Reape und McCabe, 2008; Yao *et al.*, 2004). Not only are mitochondria one of the main producers of reactive oxygen species (ROS), which could be triggers for senescence, but very importantly they also provide ATP and carbon skeletons from the tricarboxylic acid (TCA) cycle to facilitate release and relocation of nutrients and degradation of proteins and cell structures. Thus, it is tempting to speculate that the observed increase in mitochondrial gene copy numbers paralleling early leaf senescence comes along with higher mitochondrial activity. Therefore, transcript accumulation of the four analyzed genes was monitored. The

determined steady-state levels showed some alternations during the different developmental stages, but did not reflect the increase in gene copy numbers between Ros50/4 and Ros50/6 (3.3.2, Fig 26). Transcript levels were found to be generally higher in young cotyledons, corresponding to some extent to gene copy numbers, which also tended to be higher in cotyledons (see 3.3.1, Fig. 23). However, no obvious tendency towards a significant increase in leaves with beginning senescence, as it was found on the DNA level, could be observed. However, it is until now unclear, whether mitochondrial gene expression is regulated on the transcriptional level at all. It was found recently that mitochondrial transcription remains unaffected by sugar starvation, whereas altered expression of nuclear components of the ATPase regulated the assembly of new complexes (Giegé *et al.*, 2005). Tissue-specific differences in the expression of mitochondrial genes are most likely mainly due to posttranscriptional processes (Smart *et al.*, 1994) and transcript stability is thought to play a very important role. The qPCR measurements reflect steady-state mRNA levels that are determined by the rate of transcription and the stability of the resulting transcripts. It is therefore conceivable that transcript stability is reduced at the onset of senescence and mitochondrial gene copy numbers are increased as a result of the decreased stability. A higher number of gene copies would lead to a higher rate of transcription that could compensate the decrease in transcript stability. Run-on analyses of mitochondrial transcription during leaf development and measurement of protein levels will help to further clarify the processes that lead to elevated gene copy numbers in senescing rosette leaves.

One of the main functions of mitochondria is the production of energy in form of ATP by oxidative phosphorylation through an electron transport chain (Saraste, 1999). This mitochondrial respiration can be measured easily by analyzing the O_2-consumption of leaves or tissues (Kurimoto *et al.*, 2004; Yoshida *et al.*, 2007). Correlations between respiration activity and the number of mitochondrial gene copies have been shown in germinating cotyledons (Breidenbach *et al.*, 1967). In the present study it was therefore tested if respiration rate increases when leaves get senescent. To this end leaves of different ages were analyzed, starting with 5- and 10-day-old cotyledons through senescent leaves of 40- and 50-day-old plants (see 3.3.3). Using rosette leaves from different age stages it could be confirmed that gene copy numbers of the four analyzed genes increased during early senescence (3.3.3, Fig. 26). Additionally, it could be approved that copy numbers in cotyledons were relatively high. Total numbers of mitochondrial gene copies were in the same range between both analyses (3.3.1, Fig. 23 and 3.3.3, Fig. 26), although they slightly differed, most probably due to differing growth conditions and selected developmental

stages. However, the phenomenon of elevated gene copy numbers during early senescence was obviously reproducible.

Surprisingly, the monitored O_2-consumption rate did not show a positive, but rather a negative correlation of increasing gene copy numbers and mitochondrial respiratory activity (3.3.3, Fig. 26). Respiration was highest in young rosette leaves and declined towards senescence. Hence, respiration obviously does not increase as cause or result of a raised number of mitochondrial gene copy numbers during early leaf senescence. This is in line with observations made previously in *Arabidopsis* leaves undergoing dark-induced senescence. Oxygen consumption likewise decreased drastically during senescence in these leaves (Keech *et al.*, 2007). The same study showed that the number of mitochondria decreases during dark-induced senescence, while the ATP/ADP ratio was constant or even increased (Keech *et al.*, 2007). This increase could be attributed to higher respiratory activity of the remaining mitochondria to compensate the lack of energy resulting from the degradation of the photosynthetic apparatus (Keech *et al.*, 2007; Keskitalo *et al.*, 2005). The effects observed during dark-induced senescence cannot be adopted one-to-one to age related senescence; however, it is possible that similar reactions take place in the leaves analyzed here. High rates of mitochondrial respiration during leaf senescence have been shown previously in different species (Collier und Thibodeau, 1995; Dizengremel und Tuquet, 1984; Satler und Thimann, 1983). However, the mitochondrion fulfills a lot of additional functions, such as the synthesis of various important molecules and complexes, including Fe-S clusters, vitamin co-factors and fatty acids (Baker *et al.*, 2006; Ewald *et al.*, 2007; Kushnir *et al.*, 2001; Millar *et al.*, 2003; Picciocchi *et al.*, 2003; Ravanel *et al.*, 2001; Yasuno und Wada, 2002). In further studies it should thus be analyzed if any metabolic changes accompany the increased gene copy numbers.

The number of gene copies determined in leaves of different ages (3.3.1, Fig. 23) lay between 50 and 150 for the most part. Copy numbers of *atp1* reached a maximum of approximately 280 in 31-day-old rosette leaves and the amount of *nad6* copies was down to a minimum of around 40 in stem leaves. The typical *Arabidopsis* mesophyll cell has been shown to contain around 600 discrete mitochondria, depending on the physiological state of the cell (Sheahan *et al.*, 2005; Stickens und Verbelen, 1996). This is in line with the observed numbers of mitochondria in other species like *Medicago truncatula*, where 500-600 discrete organelles were detected (Sheahan *et al.*, 2004). Thus, the data presented here clearly shows that leaf cells of *Arabidopsis* at all analyzed points of development contain less copies of mitochondrial genes than mitochondria. It has been shown previously that

mitochondria can contain less DNA than expected based on their genome size (Bendich und Gauriloff, 1984; Fujie *et al.*, 1993; Kuroiwa *et al.*, 1992; Lonsdale *et al.*, 1988; Satoh *et al.*, 1993; Suzuki *et al.*, 1996; Takanashi *et al.*, 2006). Furthermore, it has been suggested that the number of mitochondria in some *Cucurbitaceae* may exceed the number of mitochondrial genomes per cell (Bendich und Gauriloff, 1984). Our data implicitly support this observation by showing total copy numbers of representative mitochondrial genes per cell for the first time with a very precise, quantitative method. Takanashi *et al.* (2006) calculated that the estimated amount of DNA in all observed mitochondria of rice roots was smaller than a full chondrome (Takanashi *et al.*, 2006). This is in agreement with the data presented here, where very low copy numbers are shown throughout leaf development, suggesting probably all mitochondria to possess less than a complete chondrome.

It has been shown by several studies that mitochondria in meristematic tissues, such as the root tip and the shoot apical meristem, contain vastly more DNA than those in more differentiated tissue, where also high amounts of mitochondria without visible nucleoids have been observed (Fujie *et al.*, 1994; Fujie *et al.*, 1993; Kuroiwa *et al.*, 1992; Takanashi *et al.*, 2006). It was suggested that mitochondria in meristematic cells contain a lot of DNA, because the rate of synthesis is high (Kuroiwa *et al.*, 1992; Sakai *et al.*, 2004), whereas the amount of mtDNA is gradually reduced when cells mature, due to continuous fission in combination with low rates of replication of the chondrome. In the present study it was found that very young cotyledons contain slightly higher amounts of mitochondrial gene copies (3.3.1, Fig. 23). This could be explained by a higher number of meristematic cells in this very young tissue.

It has been proposed that this shortage of mtDNA in higher plants is compensated by frequent fusion of mitochondria (Arimura *et al.*, 2004; Lonsdale *et al.*, 1988), which allows for an exchange of internal molecules and especially for recombination of the chondrome (Lonsdale *et al.*, 1988). It could be an evolutionary advantage for plants to be able to save energy and resources normally needed for replication of DNA and use it to grow instead. Recombination in higher plant mitochondria takes places at large and small repeated sequences and leads to a highly complex organization of the chondrome, which is composed of a heterogeneous population of small circular and large, circularly permuted molecules (Andre *et al.*, 1992; Backert *et al.*, 1995; Bendich, 1993; Bendich, 1996; Lonsdale *et al.*, 1988). These subgenomic and sometimes also substoichiometric molecules may even replicate autonomously (Abdelnoor *et al.*, 2003; Lonsdale *et al.*, 1988; Small *et al.*, 1989). Due to this organization it has been suggested previously that the chondriome (all

mitochondria within a cell) and the mtDNA population of a cell must be considered as single entities that form a "discontinuous whole" (Logan, 2006; Lonsdale *et al.*, 1988).

In the present study it has been shown that the copy numbers of different mitochondrial genes per cell differ to some extent (3.3.1, Fig. 23; 3.2.3, Fig. 22). Unequal PCR efficiencies could be ruled out as reason for the observed differences (3.3.1, Fig. 24). Statistical analyses showed that gene copy numbers of *atp1* differed substantially from those of *cox1*, *nad6* and *rps4* throughout leaf development, whereas almost no significant differences where found between the copy numbers of *rps4* and *nad6* (Fig. 3).

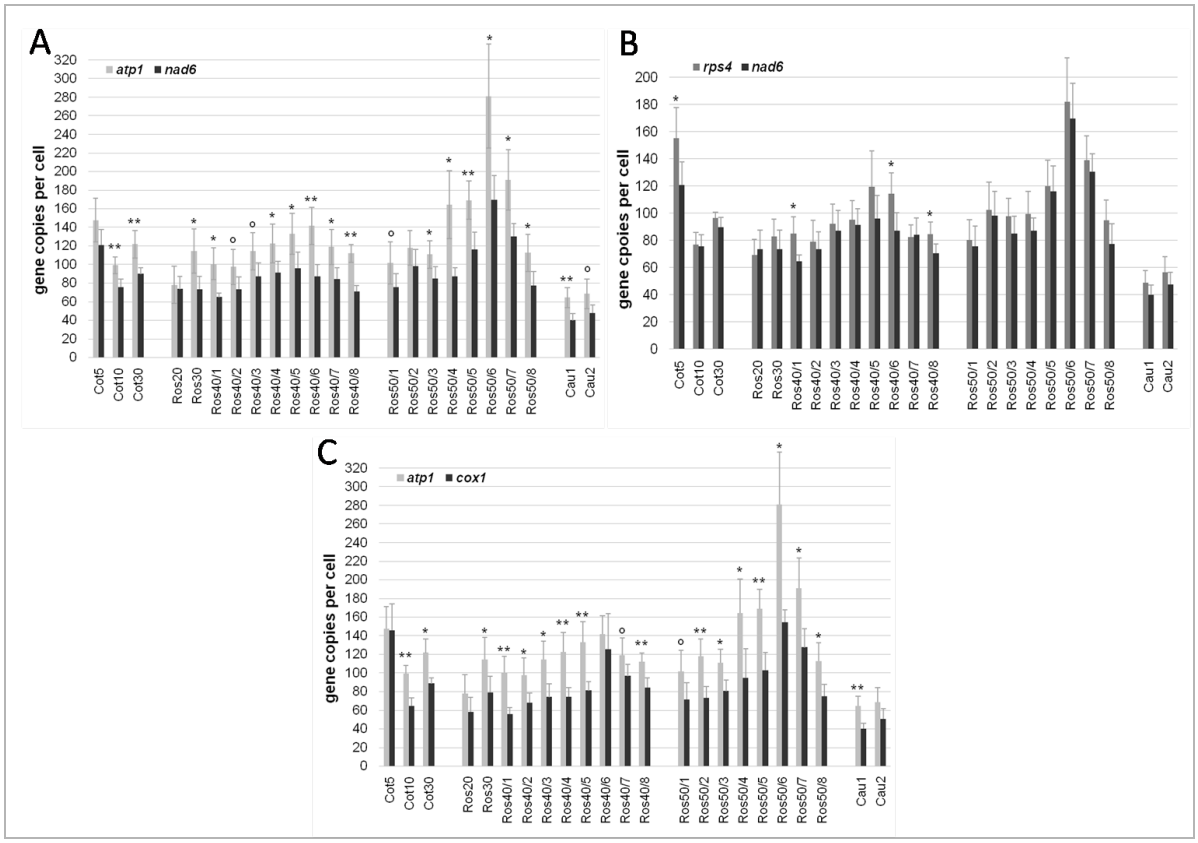


Figure 3: Statistical analysis of the differences between gene copy numbers of different mitochondrial genes during *Arabidopsis* leaf development.
The t-test (using the GraphPad InStat v 3.05 software) was applied to detect significant differences between the copy numbers of two genes. (** = $p<0.01$; * = $p<0.05$; ° = $p<0.1$)

The plastome most probably exists as circular chromosomes (Krause, 2008; Wakasugi *et al.*, 2001) and is replicated on the whole (Heinhorst und Cannon, 1993; Kunnimalaiyaan und Nielsen, 1997). It is thus to be expected that copy numbers of different genes do not differ from each other, because they should be replicated equally. Indeed, it has recently been shown that copy numbers of plastid genes did not differ significantly from each other

throughout leaf development in *Arabidopsis thaliana* (Zoschke *et al.*, 2007). The fact that different mitochondrial genes show unequal copy numbers suggests that they are replicated autonomously. In fact, the mitochondrial genome supposedly does not exist as a circular "master chromosome" (Lonsdale *et al.*, 1988), but rather comprises a very heterogeneous population of subgenomic molecules that result from frequent recombination events at repeated sequences and probably replicate autonomously afterwards (Abdelnoor *et al.*, 2003; Backert und Börner, 2000; Bendich, 1996; Fauron *et al.*, 1995; Oldenburg und Bendich, 1996; Small *et al.*, 1989). The quantitative data presented here clearly evidence that at least some of the analyzed genes reside on different subgenomic molecules and are thus present in different numbers. In particular *atp1* is shown to be present in higher numbers than all other genes tested in almost all samples (Fig. 3A and C). Interestingly, copy numbers of *rps4* and *nad6* did not differ significantly at all, except for very few samples (Fig. 3B). These two genes were chosen intentionally because they are located adjacent on the chondrome. The finding that their copy numbers do not differ further supports the hypothesis of genes residing different subgenomic molecules. It is unlikely that adjacent genes would be separated very frequently to be replicated autonomously on subgenomic molecules. In the case of *atp1* on the other hand it very probable, because *atp1* on the chondrome is very much apart from the other tested genes. In preliminary experiments using reverse PCR it has been shown that *atp1* indeed resides on a DNA fragment of defined size in all analyzed samples ranging from 7-day-old seedlings to leaves of different ages and even root samples (data not shown).

In further experiments, structure and composition of subgenomic molecules containing the analyzed genes should be investigated to get more precise knowledge of their size. Additionally, it would be very interesting to see if certain genes always reside on defined subgenomic molecules or if recombination processes produce random molecules. This knowledge would very much improve our understanding of the organization of the mitochondrial genome.

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Abbreviations

°C	**degrees Celsius**
3D	**three-dimensional**
A, C, G, T, U	nucleid acid bases (adenine, cytosine, guanine, thymin, uracil)
ADP	adenosine diphosphate
APS	ammoniumperoxodisulfate
Asp	aspartic acid
(d)ATP	(deoxy)adenosine triphosphate
BSA	bovine serum albumin
bp	base pairs
cDNA	complementary DNA
cpDNA	chloroplastidial DNA
cm	centimeter
CTAB	cetyl trimethyl ammonium bromide
dCTP	deoxycytidine triphosphate
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucelic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
e.g.	*exempli gratia* (for example)
et al.	et altera (and others)
EtBr	ethidium bromide
EtOH	ethanole
Fe-S	iron-sulfur
Fig	figure
g	gram
gDNA	genomic DNA
GFP	green fluorescent protein
dGTP	deoxyguanosine triphosphate
GUS	β-glucuronidase
h	hour
HCl	hydrochloric acid
i.e.	*id est* (that is)
kDa	kilodalton
kbp	kilobase pairs
KOH	potassium hydroxide
kPa	kilopascal
L	liter
LED	light emitting diode
m	meter
M	molar
mg	milligram
µg	microgram
Mg	magnesium
mm	millimeter
µm	micrometer
µM	micromolar
µmol	micromol
min	minute
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide

NEP	nuclear encoded plastid RNA polymerase
nm	nanometer
NTC	no template control
NUMT	nuclear copies of mitochondrial genes
MOPS	morpholinopropan-sulfonic acid
mRNA	messenger RNA
mtDNA	mitochondrial genome
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	plastid encoded plastid RNA polymerase
pH	*potentia hydrogenii*, -log [H+]
pmol	picomol
PMSF	phenylmethyl sulphonyl fluoride
RNA	ribonucleic acid
RNAP	RNA polymerase
ROS	reactive oxygen species
rpm	rounds per minute
rRNA	ribosomal RNA
RT	reverse transcriptase
s	second
SDS	sodium dodecyl sulfate
sol.	solution
TAC	transcriptionally active chromosome
TEMED	NNN'N'-tetramethyl-ethylenediamine
Tic	translocase of the inner plastid membrane
TIM	translocase of the inner mitochondrial membrane
Toc	translocase of the outer plastid membrane
TOM	translocase of the outer mitochondrial membrane
Tris	tris (hydroxymethyl)-aminomethane
tRNA	transfer RNA
dTTP	deoxythymidin triphosphate
UV	ultra violet
V	Volt
v/v	volume per total volume
vol	volume
w/v	weight per volume

Awards, Publications and Conference Abstracts

AWARDS

Eppendorf PostDoc Award, XII. Jahrestagung der ISE-G, Martinsried, Germany

PUBLICATIONS

Preuten, T., Swiatecka-Hagenbruch, M., Liere, K. and Börner, T. (2009) *Influence of light on the expression of plastidial phage-type polymerases and plastidial gene expression in* Arabidopsis thaliana*.* Manuscript in preparation.

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T. Preuten (2004) *Phage-type RNA-Polymerases and putative specificity factors: Analysis of light-dependent gene expression.* Havel Spree-Kolloquium, Berlin, Germany

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T. Preuten (2008) *Klein aber fein - Das mitochondriale Genom von* Arabidopsis – *geringe Kopienzahlen und hohe Variabilität*. XII. Jahrestagung der ISE-G, Martinsried, Germany

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T. Preuten, R. Zoschke, K. Liere and T. Börner (2008) *Good things come in small packages – the* Arabidopsis *mitochondrial genome has few copies but high variability*. Gordon-Kenan Graduate Research Seminar and Gordon Conference on Mitochondria and Chloroplasts, Biddeford, Maine, USA

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Eidesstattliche Erklärung

Hiermit versichere ich, die vorliegende Dissertation eigenständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die dem Verfahren zugrunde liegende Promotionsordnung ist mir bekannt.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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